Influence of interspecific plant interactions on the stress response of grassland species in a future climate

Proefschrift ingediend tot het behalen van de graad van doctor in de wetenschappen: biologie aan de Universiteit Antwerpen te verdedigen door

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Antwerpen 2017

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Date of public defence: 19 April 2017

Van De Velde, H. 2017. Influence of interspecific plant interactions on the stress response of grassland species in a future climate. PhD thesis. University of Antwerp, Antwerp (Belgium).

The research reported in this thesis was conducted at Centre of Excellence PLECO (Plants and Ecosystems), Department of Biology, University of Antwerp and at the Terrestrial Ecology Unit, Department of Biology, Ghent University.

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This research was funded by a grant of the Research Foundation – Flanders (FWO).

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INFLUENCE OF INTERSPECIFIC PLANT INTERACTIONS ON THE STRESS RESPONSE OF GRASSLAND SPECIES IN A FUTURE CLIMATE

INVLOED VAN INTERSPECIFIEKE PLANTINTERACTIES OP DE STRESSRESPONS VAN GRASLANDSOORTEN IN EEN TOEKOMSTIG KLIMAAT

Proefschrift voorgelegd tot het behalen van de graad van doctor in de wetenschappen: biologie aan de Universiteit Antwerpen en de Universiteit Gent te verdedigen door

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1 GENERAL INTRODUCTION

Helena Van De Velde

1.1 CLIMATE CHANGE

Since the industrial revolution, the atmospheric concentration of the main greenhouse gas, atmospheric carbon dioxide (CO_2), has increased from a pre-industrial level of 280 ppm to approximately 400 ppm at present due to anthropogenic effects, i.e. fossil fuel combustion, deforestation and biomass burning (Andreae & Merlet, 2001; Keeling *et al.*, 2009; IPCC, 2014). This increase in CO_2 is causing an exceptional rate of planetary warming. Observations have shown that over the period 1880 to 2012, global surface temperatures have risen by 0.85 °C (IPCC, 2014). Current rates of fossil fuel use will cause further warming in the 21ste century. Based on different socio-economic scenarios, Earth System Models predict a further rise of CO_2-equivalent concentrations to values between 430 and 1230 ppm by the end of the century, contributing to expected increases in global average surface temperature of 0.3 – 4.8 °C over the same period (Fig. 1, Fig. 2) (IPCC, 2014).

Climate models, however, not only predict gradual climate changes. The frequency and intensity of extreme events are expected to change as well with the Earth's climate change, and these changes could occur even with relatively small mean climate changes (IPCC, 2014). In a future climate, there will be an increased risk of more intense, more frequent and longer-lasting heat waves because a shift in the mean temperature gives rise to pronounced changes in the tails of the probabilistic distributions (Schar *et al.*, 2004; Fischer & Schar, 2009; IPCC, 2014). Several well documented heat waves have occurred during the past years such as those of 2003 (Europe), 2010 (Russia) and 2012 (North America). The likelihood of such major events is expected to increase 5 to 10-fold within the next 40 years (Barriopedro *et al.*, 2011). In a warmer future climate, most models project increased summer dryness which indicate a greater risk of summer drought (IPCC, 2014). Also, in a warmer world, alterations in patterns of global air circulation and in the hydrologic cycle will affect global and regional precipitation patterns, and increase the frequency and magnitude of droughts and floods (Easterling *et al.*, 2000; IPCC, 2014).

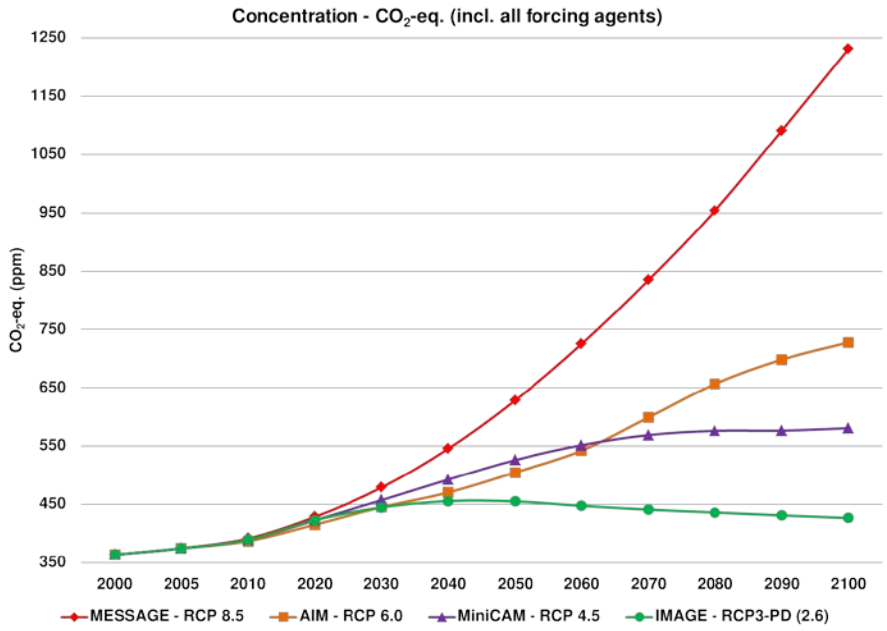


Fig. 1 The expected buildup of greenhouse gases in the atmosphere (in units of carbon dioxide equivalent greenhouse forcing) according to four Representative concentration pathways (RCP). RCPs represent atmospheric greenhouse gas concentration trajectories adopted by the Intergovernmental Panel on Climate Change (IPCC) for its fifth Assessment Report in 2014. Higher concentrations will result from higher emissions and a lack of action to curtail emissions, whereas lower concentrations may result from lower economic growth or active efforts to reduce greenhouse gas emissions. RCP units are watts per square meter, corresponding to the radiative forcing of various concentrations in 2100 (adapted from Hannah 2014).

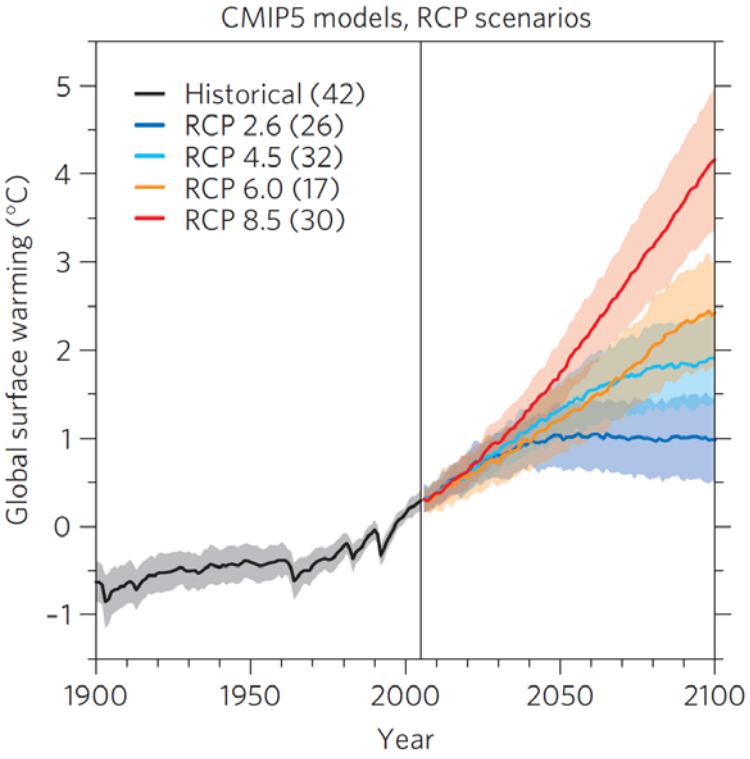


Fig. 2 Global temperature change (mean and one standard deviation as shading) relative to 1986-2005 for the Representative Concentration Pathways (RCP) used by general circulation models (adapted from Knutti and Sedlacek 2013).

1.2 STRESS

Wherever they grow, plants are continuously exposed to a broad range of environmental stressors. As sessile organisms, plants cannot run away from a stressful situation. Stress can be defined as an environmental abiotic or biotic factor that reduces the rate of some physiological process (e.g. growth or photosynthesis) below the maximum rate that the plant could otherwise sustain (Lambers *et al.*, 2008). Examples of abiotic environmental stressors include excessively high or low temperatures, drought, low nitrogen availability and high salinity. Biotic environmental stress, resulting from interactions with other organisms, is mainly common in dense plants stands and wherever plants are intensively used by animals (e.g. grazing and trampling) and microorganism (e.g. viruses, bacteria and fungi) (Larcher, 2003).

Plants respond immediately to stress by reducing their performance. Stress disrupts normal structures and the coordination of various processes at the molecular, cellular and entire organism levels (Larcher, 2003). Cellular responses to stress include changes in the cell cycle and cell division, changes in the endomembrane system and vacuolization of cells, and changes in the cell wall architecture, all leading to enhanced stress tolerance of cells (Taiz & Zeiger, 2002). At the level of plant metabolism, the amount of reactive oxygen species (ROS) generally increases as a result of stress-induced perturbations (Mittler, 2002). These ROS can act as signals for the activation of stress response and defence pathways but they can also be harmful for biological structures and processes (Asada, 1999). Plants have developed a robust antioxidant defensive system to minimize the oxidative effects of ROS and protect their basic functioning (Mittler *et al.*, 2004). However, restabilising and reparative reactions, required for the survival of stress conditions, demand additional energy and metabolites, often at the expense of biomass production, growth and reproductive efficiency (Larcher, 2003). At a certain level of stress, plants may pass their thresholds of acclimation and adaptation, which may result in mortality (Niu *et al.*, 2014).

1.3 STRESS AND CLIMATE CHANGE

Although a large body of literature exists on the stress responses of plants, much less is known about how plants will be affected by stress factors in a future climate with more atmospheric CO_2 and higher air temperatures. Will plants that grow in a modified climate still exhibit the same stress response? Reduced stress resistance of plants in a future climate might lead to the disruption of community interactions and an altered community composition due to the loss of the most sensitive species. In turn, compositional and diversity changes in plant communities can alter ecosystem structure and function (Wardle *et al.*, 2000; Hooper *et al.*, 2005).

On the one hand, climate change can alter the intensity of a stressor. For instance, herbivory rates are expected to increase exponentially with rising temperature (Gillooly *et al.*, 2001; O'Connor, 2009; O'Connor *et al.*, 2011) because ectothermic herbivores must increase food intake at higher temperatures to offset increased metabolic or nutritional demand (O'Connor *et al.*, 2001). On the other hand, the plant's intrinsic stress response might be altered too. Elevated CO_2 and warming may affect the secondary metabolite productions which are well-known for their role in plant defence against insect herbivory (Robinson *et al.*, 2012; Pellissier *et al.*, 2014). This thesis focuses on the plant responses to the abiotic stressors heat and drought and the biotic stressor herbivory in a future climate with elevated CO_2 and warming. In the next sections, I first describe some general plant responses to climate change in order to better understand how climate change may alter the plant's intrinsic stress response. I focus on the responses of plant biomass and metabolites. However, elevated CO_2 and warming have been shown to affect other aspects of plants such as respiration, photosynthesis, water/nutrient acquisition and phenology. More details can be found in Ainsworth and Long (2005), Leakey *et al.* (2009), Newman (2011), Rustad *et al.* (2001), Soussana and Luscher (2007) and Wu *et al.* (2011). Second, I describe the possible impact of climate change on the plant responses to heat, drought and herbivory stress.

1.4 PLANT RESPONSES TO CLIMATE CHANGE

1.4.1 Carbon dioxide enrichment

Over short periods of time, elevated CO_2 can stimulate plant growth directly through enhanced photosynthesis, or indirectly through its effect on the hydrological cycle as elevated CO_2 decreases stomatal conductance, leading to increased water use efficiency (Bazzaz, 1990; Long *et al.*, 2004; Morgan *et al.*, 2004). However, over longer timeframes, plants may downregulate photosynthetic activity through physiological and biochemical adjustments or resource limitations (Ainsworth & Long, 2005; Crous *et al.*, 2010; Reddy *et al.*, 2010; Lee *et al.*, 2011).The stimulation of photosynthesis causes a number of changes in plant primary metabolism. Hence, plants exposed to a CO_2-enriched environment show higher concentrations of carbohydrates (including starch and soluble sugars) and lower concentration of nitrogen (either from dilution by increased carbohydrates or reallocation) (Lincoln *et al.*, 1993; Bezemer & Jones, 1998; Stiling & Cornelissen, 2007; Robinson *et al.*, 2012). As a result of increased carbohydrates and reduced protein levels, the C:N ratio increases under elevated CO_2 (Robinson *et al.*, 2012).

In addition, elevated CO_2 may also cause changes in plant secondary chemistry, which have been frequently explained on the basis of the carbon-nutrient hypothesis (Bryant *et al.*, 1983). This hypothesis predicts that carbon-based defence compounds such as phenolics and terpenoids will increase as a result of the 'excess' carbon under elevated CO_2, and that nitrogen-based defence compounds such as alkaloids, cyanogenic glycosides and glucosinolates will decrease as a result of scarce nitrogen (Bryant *et al.*, 1983). Indeed, elevated CO_2 tends to increase phenolics, flavonoids and tannins and decrease nitrogen-containing plant defences, but the responses of these and other secondary compounds are highly variable (Robinson *et al.*, 2012; Zavala *et al.*, 2013). In conclusion, changes in primary metabolism as a result of rising atmospheric CO_2 levels are relatively predictable, but changes in plant secondary metabolism are highly variable and needs further investigation.

1.4.2 Temperature

Warming increases photosynthesis as long as the plant's optimal temperature is not exceeded and in the absence of photosynthetic acclimation (Berry & Bjorkman, 1980). Therefore warming can stimulate plant biomass production via higher photosynthesis and/or mineralization rates (Hartley *et al.*, 1999; Rustad *et al.*, 2001; Penuelas *et al.*, 2007; Wu *et al.*, 2011). By contrast, other studies have shown that warming retards plant biomass production and photosynthesis due to warming induced drought and heat stress (De Valpine & Harte, 2001; De Boeck *et al.*, 2008; Sherry *et al.*, 2008).

The net effect of temperature on the concentration of soluble sugars depends on whether photosynthesis is operating below or above its thermal optimum (DeLucia *et al.*, 2012). Furthermore, the response of leaf nitrogen to warming is inconsistent. Studies have shown that warming can either decrease (An *et al.*, 2005; Flynn *et al.*, 2006; Jamieson *et al.*, 2015) or increase leaf nitrogen concentrations (Volder *et al.*, 2015). In a review, Sardans *et al*. (2012) concluded that warming can increase, decrease, or have no effect on the C:N ratios of plants depending on the type of plant and the climate where it grows (Sardans *et al.*, 2012).

Compared with primary metabolites, less is known about the effect of temperature on the concentration of secondary metabolites in plants (Bidart-Bouzat & Imeh-Nathaniel, 2008). According to the growth-differentiation balance hypothesis (Herms & Mattson, 1992), warming-accelerated photosynthesis should contribute to growth rather than defence if resources (e.g. soil moisture and nutrients) are not limiting, and thus levels of carbon-based secondary metabolites should decline. However, so far, studies have demonstrated that warming has variable effects on different groups of chemical defences (Zvereva & Kozlov, 2006). For example, terpenoids increase under the impact of warming, while phenolic constituents, such as flavonoids and tannins, tend to decrease with warming (Zvereva & Kozlov, 2006; Bidart-Bouzat & Imeh-Nathaniel, 2008). Such variation in the effect of temperature on the chemical composition of plants makes it difficult to predict how climate change will alter plant resistance. Hence, there is an urgent need for more research on the effect of warming on phytochemistry.

1.5 HEAT STRESS – IMPORTANCE OF LEAF TEMPERATURE

As mentioned earlier, besides moderate warming, the current climate change is increasing both the likelihood and the intensity of extreme climate events such as heat waves. The 40 to 50 °C temperature range is a general threshold for heat stress in plants across almost all biomes (Larcher, 2003). Such excessive temperatures cause an array of morpho-anatomical, physiological and biochemical changes in plants, which ultimately reduce growth and economic yield (Wahid *et al.*, 2007; Bastos *et al.*, 2014). Today, experimental studies and models that examine or try to predict the impact of heat waves, focus on air temperatures (Bauweraerts *et al.*, 2013; Sentis *et al.*, 2013; Deryng *et al.*, 2014) while leaf temperatures are a better indicator of heat stress because plant physiological processes and metabolic rates are affected by leaf temperatures rather than air temperatures. Leaf temperature not only has an effect on plant metabolic processes but also on folivorous insects that are in intimate contact with leaves (Pincebourde & Casas, 2006). For instance, the leaf temperature affects the consumption rate of insect herbivores (Zavala *et al.*, 2013). Leaf temperatures are determined by the stomatal response of the plants and a number of environmental conditions such as radiation, wind speed, air humidity and air temperatures (Campbell & Norman, 1998; Jones, 2013).

1.6 IMPACT OF CLIMATE CHANGE ON DROUGHT RESPONSE OF PLANTS

Drought is one of the major limitations for plant productivity, mainly through decreased stomatal conductance and down-regulation of photosynthetic machinery and/or increased allocation to the roots (Chaves *et al.*, 2002). Although plant responses to drought stress have been studied intensively (e.g. Chaves *et al.*, 2002; Farooq *et al.*, 2009), little work has been done to investigate the interactions of drought stress with elevated CO_2 and high air temperatures. Nevertheless, elevated CO_2 and warming might affect the plant responses to drought stress. As mentioned earlier (see above), warming can stimulate plant biomass production via higher photosynthesis and/or mineralization rates (Rustad *et al.*, 2001; Wu *et al.*, 2011), but can deteriorate it via associated drought stress and heat (De Boeck *et al.*, 2008; Sherry *et al.*, 2008). These associated stresses result from initially enhanced soil water depletion as warming almost always lowers soil moisture (Rustad *et al.*, 2001; Zavaleta *et al.*, 2003) by increased evapotranspiration (Allen *et al.*, 2003). Similarly, warming is expected to aggravate drought stress. Elevated CO_2 decreases stomatal conductance and consequently increases water use efficiency and soil water availability (Long *et al.*, 2004; Morgan *et al.*, 2004; Ainsworth & Long, 2005; Leuzinger & Korner, 2007). Hence, elevated CO_2 could alleviate the deleterious effect of drought through indirect effects on water consumption.

Elevated CO_2 may affect the drought stress-induced ROS-levels and antioxidant defence system (AbdElgawad *et al.*, 2016). It may reduce the ROS formation and thus diminish intrinsic oxidative stress, which may result from decreased photorespiration (Ainsworth *et al.*, 2008; Zinta *et al.*, 2014; AbdElgawad *et al.*, 2015). Consequently, this can lead to a down-regulation of the antioxidant defence system under elevated CO_2 (Erice *et al.*, 2007). However, other studies have found an enhanced protective capacity (Schwanz & Polle, 2001; Zinta *et al.*, 2014). In addition, it has been shown that warming may enhance the drought induced oxidative stress (Farfan-Vignolo & Asard, 2012) and possibly it activates the antioxidant defence system (Han *et al.*, 2009; Wang *et al.*, 2014).

1.7 IMPACT OF CLIMATE CHANGE ON PLANT-HERBIVORE INTERACTION

1.7.1 Carbon dioxide enrichment

While it is believed that increased CO_2 has little direct effect on insect herbivores performance (Pritchard *et al.*, 2007), few studies account for such direct effects (but see Awmack *et al.*, 1997; Stange, 1997). Therefore, any change is most likely to be caused indirectly via changes in the quality of the host plant as a food source. Elevated CO_2 induces changes in morphology, physiology and plant chemistry that are likely to affect the nutritional quality of host plants for insect herbivores (Fig. 3) (Robinson *et al.*, 2012). For instance, elevated CO_2 reduces foliar nitrogen concentration, alters leaf toughness and concentrations of carbon- and nitrogen-based secondary metabolites (Fig. 3) (Lincoln *et al.*, 1986; Bidart-Bouzat & Imeh-Nathaniel, 2008; Cornelissen, 2011; Robinson *et al.*, 2012). Low nitrogen concentration means a lowered concentration of leaf protein and amino acids and, as a consequence, reduced nutritive value to herbivores (Lincoln *et al.*, 1986). Insect herbivores respond to these low-quality plants by increasing food consumption to compensate for the plant's lowered nutritional quality, by reducing their growth rates, by prolonging their development time, and by reducing food conversion efficiency (Stiling & Cornelissen, 2007; Robinson *et al.*, 2012). Reduced growth rates result in lower pupal and adult weights (Robinson *et al.*, 2012). In general the fitness of insect herbivores reduces under CO_2 enrichment (Lincoln *et al.*, 1993; Bezemer & Jones, 1998; Hunter, 2001), which would have the potential to increase mortality imposed by natural enemies (Stiling *et al.*, 2003). This in turn would reduce herbivore abundance, richness and diversity if compared to ambient CO_2 conditions (Fig. 3) (Stiling & Cornelissen, 2007; Cornelissen, 2011).

The response of insect herbivores to CO_2 enrichment as described above are on average, there is significant variation among arthropods orders and feeding guilds (e.g. chewers versus sap suckers). Today, the majority of studies have been biased to free-feeding herbivores and many more studies are necessary to obtain a clearer pattern of CO_2 effects on other guilds of herbivores.

The compensatory feeding hypothesis, in which increased feeding compensates for poor nutritional quality (Lincoln *et al.*, 1986; Schaedler *et al.*, 2007), do not predict the diverse responses of insects to food sources developed under elevated CO_2. Studies have shown that compensatory feeding is not enough to fully compensate for reduction in food quality, indicating that this altered behavioural strategy is not without a cost, particularly at early stages of larval development (Fajer *et al.*, 1989; Bezemer & Jones, 1998; Johnson & McNicol, 2010; Johnson *et al.*, 2014). Increased consumption of secondary metabolites, which play a crucial role in plant defence against herbivores, may be a reason for this (Zavala *et al.*, 2013). The concentration of these compounds, and the hormones responsible for inducing their production, are themselves affected by elevated CO_2 (DeLucia *et al.*, 2012; Robinson *et al.*, 2012; Zavala *et al.*, 2013).

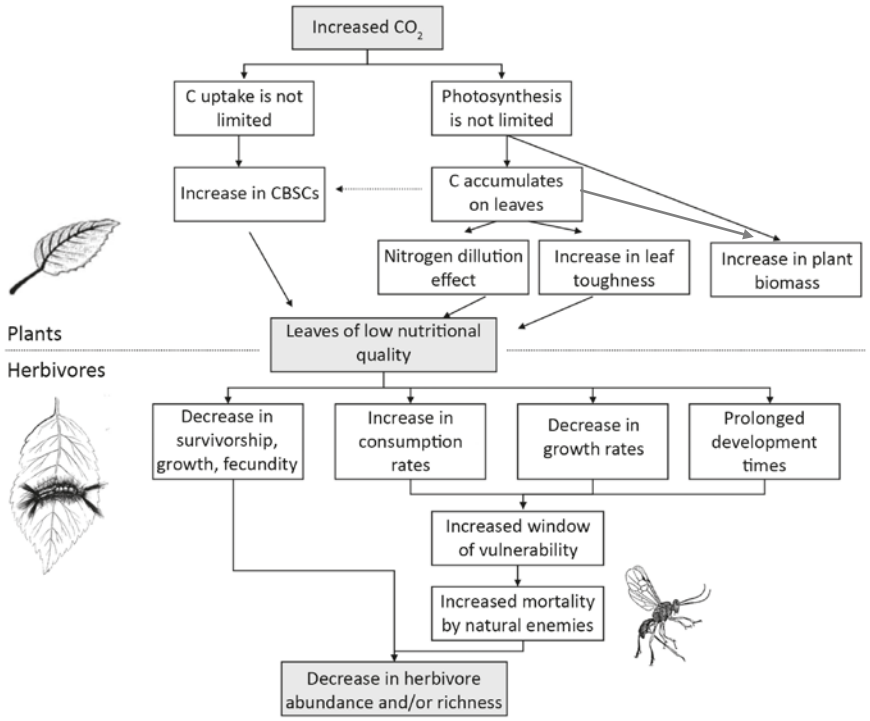


Fig. 3 Predicted effects of elevated CO_2 conditions on plants and insect herbivores (C: carbon, CBSCs: carbon-based secondary compounds) (Adapted from Cornelissen 2011).

1.7.2 Temperature

Temperature is the dominant abiotic factor for poikilothermic animals, such as insects, which have limited ability to regulate their body temperature. Therefore, their body temperature is dependent on that of their environment (Porter *et al.*, 1991) and it is this body temperature which controls their developmental and metabolic processes. Many insects live in conditions below their thermal optima; increases in temperature have been shown to shortens development time (Bale *et al.*, 2002) and increases fecundity (Meisner *et al.*, 2014) of insect herbivores until some threshold. Faster development may lead to population increases via reduced generation time and decreased exposure to natural enemies (Jamieson *et al.*, 2012). Contrary to elevated CO_2, warming has a direct positive effect on insect herbivore performance.

Warming may affect plant nitrogen content, primary and secondary metabolites (see above) and therefore warming may substantially affect plant quality. However, only a few studies have focused on indirect effects on insect herbivore performance and consequently, the net effect is difficult to predict. According to Bauerfeind and Fischer (2013), warming may reduce herbivore host-plant quality. So far, the results suggest that indirect effects are not likely to counterbalance the direct positive effects, and the overall herbivore response to warming will be positive (Zvereva & Kozlov, 2006; Bauerfeind & Fischer, 2013).

By modelling population dynamics, Newman (2004) predicted that aphids will most likely benefit from elevated CO_2 as long as nitrogen is not limiting (i.e. when soil N inputs are high and aphid N requirements are low) but this effect will be reduced when air temperature increases. So far, two empirical studies found support for these predictions using legumes as model species (Ryalls *et al.*, 2015; Ryalls *et al.*, 2017).

1.8 INTERACTIVE EFFECTS OF ELEVATED CO_2 AND TEMPERATURE

Although it is recognized that temperature and atmospheric CO_2 concentrations will increase concurrently, previous work has focused mainly on the independent effects of elevated CO_2 and warming on plant stress (Lincoln *et al.*, 1993; e.g. Hughes & Bazzaz, 2001; Llorens *et al.*, 2003; Penuelas *et al.*, 2007). However, multifactor climate change experiments are crucial for our understanding of future ecosystem functioning as plant responses to the combination of different abiotic factors are unique and cannot be directly interpreted from the single factor response (Xu *et al.*, 2013). For instance, Wu *et al.* (2011) have shown that the combined responses to warming and altered precipitation tend to be smaller than expected from additive, single-factor effects; consequently, multi-factor experiments are needed.

Today, there are only a few studies that have investigated plant responses to drought, warming and elevated CO_2 (e.g. Bloor *et al.*, 2010; Kongstad *et al.*, 2012; Naudts *et al.*, 2013). Also studies exploring plant-herbivore interaction under simultaneous increase of both temperature and CO_2 are surprisingly scarce and all of them were conducted with different insect guilds. However, different feeding guilds of herbivores respond to changes in host plant quality in different ways. Therefore, there are gaps in our general knowledge of insect response to simultaneously changing climate factors. So far, the few multifactor climate change experiments do not allow drawing general conclusions and consequently more work is needed.

1.9 IMPORTANCE OF PLANT-PLANT INTERACTIONS

A great majority of studies on climate change focus on the responses of individuals and species. As the impact of climate change at single species level is fairly well known (particularly the independent effects of elevated CO_2 and warming), an important next step is to study species responses to environmental changes in more natural conditions. In nature, species interact with many others at the same or adjacent trophic levels. Therefore responses by individual species to climate change are not isolated (Harrington *et al.*, 1999; Voigt *et al.*, 2003; Tylianakis *et al.*, 2008; Van der Putten *et al.*, 2010). Temperature or CO_2 can cause organismal or population changes. Such changes can affect other members of a community via species interactions. For instance, elevated CO_2 concentrations can have a direct impact on the relative competitive abilities of plant species. Species specific stimulation of growth by elevated CO_2 (Poorter & Navas, 2003) may alter the balance of plant-plant interactions (Brooker, 2006).

Plant-plant interactions play a key role in regulating the composition of communities and ecosystems. They control the community composition, for example through their effects on resource availability or the habitat structure (Brooker, 2006). However, the impact of plant-plant interactions can be altered by external drivers such as climatic conditions. Therefore, because of the important role of plant-plant interactions and the current speed and impact of climate change, there is an urgent need to investigate how plant-plant interactions may be playing a role in mediating the response of ecosystems to stress factors and/or drivers of climate change, and how plant-plant interactions might themselves be influenced by climate change. Climate change can modify the direction and intensity of species interactions such as plant-plant interactions and in this way enhance or counteract the direct effects of climate change (Tylianakis *et al.*, 2008).

1.10 MODEL SYSTEM

In this PhD thesis I used narrow-leaved plantain, *Plantago lanceolata L.*, its associated aboveground insect herbivore *Dysaphis plantaginea* Passerini (Hemiptera: Aphididae) and a heterospecific neighbouring plant species, perennial ryegrass, *Lolium perenne* L. as a model system to address the issues raised above.

1.10.1 Plantago lanceolata

Plantago lanceolata L. (Plantaginaceae) is a cosmopolitan, short-lived perennial forb with rosette growth form and adventitious roots that may reproduce via seeds or by vegetatively forming new rosettes from axillary buds (Sagar & Harper, 1964). *P. lanceolata* produces numerous leaves and spiked inflorescences at the end of fibrous stalks (Sagar & Harper, 1964; Cavers *et al.*, 1980). The species is self-incompatible and wind-pollinated. *P. lanceolata* has been reported to display anisohydric behaviour under drought (Van den Berge *et al.*, 2014).

Plantago lanceolata produces the iridoid glycosides aucubin and catalpol as well as a number of bioactive phenolic compounds such as flavonoids and pheylethanoid glycosides (Rønsted *et al.*, 2000; Gálvez *et al.*, 2005). Iridoid glycosides are products of the isoprenoid biosynthetic pathway (McGarvey & Croteau, 1995). They are members of a large group of terpene derivates, the iridoids (Dobler *et al.*, 2011). In *P. lanceolata*, iridoid glycosides are the primary allelochemicals found in large concentrations in both below- and aboveground tissues (Bowers *et al.*, 1992). For instance aucubin and catapol concentrations may be as high as 10-12% of dry weight which may result in high unpalatability and/or toxicity to generalist herbivores feeding on shoot and root tissues (Bowers & Stamp, 1992; Bowers & Stamp, 1993; De Deyn *et al.*, 2004). Yet, these iridoid glycosides are also used as feeding and/or oviposition stimulants by several specialist herbivores (Bowers, 1984; Pereyra & Bowers, 1988) and some have the ability to sequester these compounds from leaves and roots and use them for their own protection against natural enemies (Opitz *et al.*, 2010). Studies have shown that aucubin and catalpol concentrations vary with atmospheric carbon dioxide levels (Fajer *et al.*, 1992), herbivory (Bowers & Stamp, 1993), genotype (Fajer *et al.*, 1992) and leaf age (Bowers *et al.*, 1992; Bowers & Stamp, 1992).

1.10.2 Lolium perenne

Lolium perenne L. (Poaceae) is an important pasture, forage and turf grass in almost all temperate regions of the world. It is a perennial grass that forms dense tussocks and reproduces mainly via seeds and rarely are plantlets formed in leaf axils through clonal propagation (Beddows, 1967). Individual tillers are annual or winter-annual and die off after inflorescence development, which may be induced by short days and/or low temperatures (Cooper, 1960; Heide, 1994). *L. perenne* is a self-incompatible, wind-pollinated species. This species has been reported to display isohydric behaviour under drought (Van den Berge *et al.*, 2014). *L. perenne* is not a host plant for *D. plantaginea*.

1.10.3 Dysaphis plantaginea

The rosy apple aphid *D. plantaginea* Passerini (Hemiptera: Aphididae) is an important destructive pest of apple (*Malus domestica* Borkh.) in Europe and North America. This species has also been reported on *Plantago major, P. lanceolata, P. media, P. radicata, Jasione montana, Crataegus monogyna, Malus orientalis, M. pumila, M. sylvestris, M. zumi, M. sieversii, Pyrus communis, Pyrus mamorensis* and *Antirrhinum majus (Holman, 2009). D. plantaginea* is a specialized host-alternating, holocyclic or heteroecious species (Blommers *et al.*, 2004). It overwinters on apple trees, the primary host plant, as eggs and hatches in the spring as fundatrices that feed on flower or leaf buds. In late May, alate (winged) morphs are produced and these migrate to the obligate alternate hosts, *Plantago major* L. and *P. lanceolata* (Alford, 2014). On *Plantago* spp., they give birth to apterous (wingless) morphs that reproduce by parthenogenesis. In mid-September, after a handful of generations, the annual period of sexual reproduction starts with the appearance of winged aphids, induced by the increasing length of nights over the previous weeks (Lees, 1966; Blommers *et al.*, 2001). Winged females make their appearance first; they have to find an apple tree, where they give birth to sexual females. A few weeks later, winged males begin to appear on plantain and these migrates to the apple trees, where they mate with the now adult females, enabling these to produce fertile eggs. The common name “rosy” indicates the colour of the aphids on apple (pinkish purple); on the summer host, a yellow-green colour morph is produced.

1.11 OBJECTIVES AND OUTLINE OF THE THESIS

A first objective of this thesis was to expand the current knowledge about the effects of elevated CO_2 and temperature on stress responses of grassland species. In addition to examining the independent effects of elevated CO_2 and warming on plant stress, we were particularly interested in the combined effect of these factors. We hypothesized that combined warming and elevated CO_2 reduces the stress impact due to a better protection mechanism. Increased C availability under elevated CO_2 may result in increased supply of defence molecules. This increased protection mitigates the biomass lost in response to stress. However, to better understand the mechanisms and processes behind the observed responses of species, it is necessary to focus not only on the impact of climate change on the actors (i.e. individuals, species) in ecological networks, but also and more intensively on the strengths of the linkages between them (Walther, 2010). Climate change can modify the direction and intensity of species interactions such as plant-plant interactions and in this way enhance or counteract the direct effects of climate change. Therefore, a second objective of this thesis was to investigate whether plant-plant interactions modified the species-specific stress responses of grassland species to climate change. In this thesis, we focused on the abiotic stressor drought and the biotic stressor herbivory. A schematic overview of the different topics addressed in this thesis is given in figure 4.

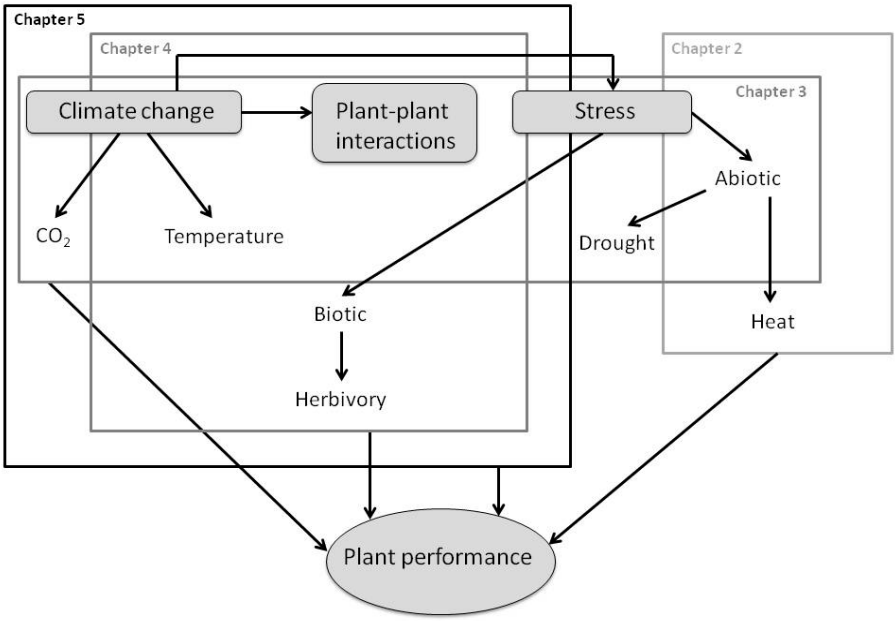


Fig. 4 Outline of the thesis indicating the three main topics: climate change, stress and plant-plant interactions. The frames represent the aspects that were studied in each chapter.

Prior to experimental studies on the topics presented above; in **chapter II**, we studied how specific environmental conditions and the plant's stomatal response affect leaf temperatures and the potential for heat stress by using both an energy balance model and field data. To examine and predict the impact of heat waves on plants, much of the focus has been on air temperatures as provided by meteorological time series. However, leaf temperature is the more relevant variable as plants' metabolic rate and physiological processes depend primarily on leaf rather than on air temperatures. A number of environmental conditions and the stomatal response of plants determine leaf temperatures. We discussed how these variables can increase or decrease the potential for heat stress during a heat wave.

In **chapter III** we focused on the abiotic stressor drought. In a multi-factorial field experiment, we investigated whether elevated CO_2 and warming could alter the drought response of plants. As drought frequency is likely to rise, understanding how fast plant communities recover from drought under elevated CO_2 and higher temperatures is necessary to determine if insufficient or compromised recovery threatens plant community stability in future conditions. Therefore, apart from focusing on the impacts of stress during the drought itself, we also examined whether drought triggers lagged effects over the growing season after the event has passed, and whether elevated CO_2 and warming alter these. To address this, we used monocultures and mixtures of two common grassland species *Lolium perenne* and *Plantago lanceolata*. Species-specific differences in responses to changing environmental conditions can alter competitive interactions within plant communities. Hence, we were also interested whether climate change factors could alter plant-plant interactions. We hypothesize that (1) warming exacerbates drought stress by decreasing the maximal photochemical efficiency and increasing dead biomass, (2) elevated CO_2 mitigates negative warming effects on drought stress by increasing photochemical efficiency and reducing biomass loss, (3) warming aggravates the lagged responses of drought caused by additional soil drought, (4) elevated CO_2 mitigates the lagged responses of drought by improved water use efficiency from stomatal closure and (5) future climate conditions would alter plant-plant interactions.

In **chapter IV**, we investigated the effect of warming on a simple model community consisting of the aphid *Dysaphis plantaginea* feeding on *P. lanceolata* and a heterospecific neighbouring plant species *L. perenne*. We used two different plant compositions, monocultures and mixtures of *P. lanceolata* and *L. perenne*. This allowed us to investigate whether warming could indirectly influence plant-herbivore interactions via effects on neighbouring plants. Several population parameters of the aphids and plant characteristics were determined in order to identify possible mechanisms. We hypothesize that (1) warming shortens the individual generation time of aphids and thus enhances the growth rate of the population, (2) warming alters the leaf nitrogen and decreases water content and thus indirectly alters the host plant quality for insect herbivores. We expect that the overall response of aphids to warming will be positive and this will cause more biomass lost at higher temperatures. Further we hypothesize that (3) interspecific competition in mixtures reduces the biomass of *P. lanceolata*. Therefore, in mixtures, *P. lanceolata* would experience more stress and be more vulnerable to aphids' attacks.

In **chapter V**, we assessed the combined effect of warming and elevated CO_2 on plant-herbivore interactions and possible indirect effects of warming and elevated CO_2 via impact on neighbouring plants. In this chapter we focused on the leaf quality, the chemical defence system and aphid performance. The same model community as in the previous chapter was used. We hypothesized that (1) combined warming and elevated CO_2 would alter foliar nutrients and defence molecules, (2) altered host quality and plant resistance would affect insect herbivore performance, (3) combined warming and elevated CO_2 indirectly influence host quality and plant resistance via effects on neighbouring plants.

In the general discussion the obtained results concerning the stress sensitivity of grassland species in a future climate are evaluated. In addition, important mechanisms for making reliable forecasts of climate change effects on grassland communities are discussed.

2 HEAT STRESS: MORE THAN HOT AIR

Adapted from: De Boeck HJ°, Van De Velde H°, De Groote T & Nijs I. (2016) Heat stress: more than hot air. Biogeosciences, 13, 5821-5825.

°: joint first authorship

2.1 ABSTRACT

Climate models project an important increase in the frequency and intensity of heat waves. In gauging the impact on plant responses, much of the focus has been on air temperatures while a critical analysis of leaf temperatures during heat extremes has not been made. Nevertheless, direct physiological consequences of heat depend primarily on leaf rather than on air temperatures. We discuss how the interplay between various environmental variables and the plants' stomatal response affects leaf temperatures and the potential for heat stress by making use of both an energy balance model and field data. The results demonstrate that this interplay between plants and environment can cause leaf temperature to vary substantially at the same air temperature. In general, leaves tended to heat up when radiation was high and when stomates were closed, as expected. But perhaps counterintuitively, also high air humidity raised leaf temperatures, while humid conditions are typically regarded as benign with respect to plant survival since they limit water loss. High wind speeds brought the leaf temperature closer to the air temperature, which can imply either cooling or warming (i.e. abating or reinforcing heat stress) depending on other prevailing conditions. The results thus indicate that heat waves characterized by similar extreme air temperatures may pose little danger under some atmospheric conditions, but could be lethal in other cases. The trends illustrated here should give ecologists and agronomists a more informed indication about which circumstances are most conductive for heat stress to occur.

2.2 INTRODUCTION

Current climate change has made heat waves more likely as both the temperature mean and variability are increasing (Schar *et al.*, 2004). Several well-documented heat waves have occurred during the past years such as those of 2003 (Europe), 2010 (Russia) and 2012 (North America), and the likelihood of such major events is expected to increase 5 to 10-fold within the next 40 years (Barriopedro *et al.*, 2011). Heat stress in plants is usually observed when tissue temperatures exceed 40 °C, a threshold that is fairly stable across biomes (Larcher, 2003). Such excessive temperatures affect plant metabolism in multiple ways, ultimately reducing growth and economic yield (Bastos *et al.*, 2014; Chung *et al.*, 2014). This seems at odds with the reported lack of significant single-factor effects in several ecological studies on heat waves (Poirier *et al.*, 2012; Hoover *et al.*, 2014; De Boeck *et al.*, 2016). We examine here how these seemingly contrasting notions can be reconciled. The fundamental issue is that air temperature (T_a) is often considered as an important indicator of heat stress, while metabolic rates and physiological processes are affected much more directly by leaf (tissue) temperatures (T_l). Many studies on heat wave effects do not measure leaf or canopy temperatures and report only on air temperatures (e.g. Bauweraerts *et al.*, 2013; Fernando *et al.*, 2014; Filewod & Thomas, 2014), which suggests an underestimation of the importance of T_l and the variables that influence it. Also in models used to predict heat stress effects, air temperatures are still often used instead of tissue temperatures, as noted by Webber *et al.* (2016) regarding crop modelling, which can lead to inaccurate predictions of crop yields (Siebert *et al.*, 2014). From literature on environmental biophysics (e.g. Campbell & Norman, 1998; Jones, 2013) we know that leaf and tissue temperatures are determined by a number of environmental conditions (apart from T_a, primarily through radiation, wind speed and air humidity) and the stomatal response of the plants. The extent to which these variables can decouple leaf from air temperatures and therefore increase or decrease the potential for heat stress during a heat wave of similar magnitude (in terms of air temperature, as it is usually considered) is discussed here by making use of both an energy balance model based on established physical equations and field data.

2.3 MATERIALS AND METHODS

The model used to calculate leaf temperature is based on the energy balance equation (Eq. 1):

$$R_{s,in} + R_{l,in} - R_{l,out} - H - \lambda E = 0 \quad (1)$$

The equation states that an equilibrium is reached under a certain set of environmental conditions (the flux of sensible heat H can be either incoming or outgoing), so that the sum of incoming energy (via shortwave radiation $R_{s,in}$ and longwave radiation $R_{l,in}$ absorbed by the leaf) and outgoing energy (outgoing longwave radiation $R_{l,out}$, and latent heat λE) is zero. The different terms are derived from other equations, which feature both environmental variables such as wind speed (u) and relative humidity (RH) of the air, leaf-scale parameters such as stomatal conductance (g_s) and characteristic leaf dimension (d), and constants such as the Stefan Boltzman's σ ($5.67e^{-8}$ W m^{-2} K^{-4}). For more details, we refer to De Boeck *et al*. (2012).

The leaf temperature is calculated in an iterative manner: as a starting situation it is assumed that leaf and air temperature are equal, in which case the energy budget equals zero. In any other situation, the model will assume T_l to be lower/higher than T_a if the energy budget is negative/positive. The iteration proceeds in a stepwise manner, until a precision of 0.01 °C is achieved. The model was validated earlier (De Boeck *et al.*, 2012), demonstrating a deviation between measured and modelled leaf temperatures of less than 1.5 °C for over 90% of the cases. The model is freely available upon request.

In this study, we set T_a at 40 °C to approximate the general threshold for heat stress. Atmospheric pressure (which has limited influence) was kept constant at 100 kPa. Emissivity, reflectivity and absorptivity parameters for leaves and soil were used like in De Boeck *et al.* (2012). In the main analyses, major inputs, namely incident shortwave energy, stomatal conductance, wind speed, and relative humidity of the air, were varied in a dichotomous manner (high or low) to clearly illustrate the direction of responses. More detailed analyses pairing input variables to better illustrate interrelations are presented as supplementary material. We focus on vegetation represented by

species that have narrow leaves (like those found in many grasses) with a characteristic dimension of 0.5 cm, but we also consider the opposite end of the spectrum, namely very broad leaves with a d of 20 cm.

The modelled results are supported by data recorded on five sunny days during a heat wave in Belgium in 2015 (1-5 July). These data were collected at an experimental site in Wilrijk, Belgium on two homogeneous 10 cm tall young grass stands sown five weeks earlier on homogenised soils (Fig. S1, see supplementary material section 1). The grass was irrigated daily (c. 5 L m^{-2}), with the exception of one day to test the impact of surface drying on the difference between T_a and T_l. Radiation sensors (SR03-05, Hukseflux Thermal Sensors, Delft, The Netherlands) had been installed approximately 30 cm above the vegetation, with one sensor directed upwards, and one sensor directed downwards to measure absorbed radiation (the difference between the two readings). At the same height, canopy temperature was recorded with a non-contact thermometer (custom made with a MLX90416ESF sensor, Melexis, Tessenderlo, Belgium). Air temperature and relative humidity were measured at 15 cm height (i.e. just above the canopy) in each plot using custom-made system (with a SHT75 RH/T_a sensor, Sensirion AG, Staefa, Switzerland) shielded from the sun by a thin wooden panel. To ensure that mostly data from times when direct sunshine reached the plots was used (generally between 9 am and 7 pm CET), we omitted data points with absorbed radiation below 100 W m^{-2}. This was done to prevent artefacts from dew or times when stomates were still closed.

2.4 RESULTS AND DISCUSSION

Our results show that high radiation loads are an important prerequisite for heat stress, unless air temperatures exceed the tissue heat stress threshold significantly. Without the energy provided by significant amounts of sunshine, plant tissues will almost always be cooler than the surrounding air, regardless of other conditions (Fig. 1, S2-4, see supplementary material section 1). In reality, heat waves usually feature clear skies (De Boeck *et al.*, 2010), implying that high radiation loads during hot weather are probable. This also means that experiments in which high air temperatures are imposed in low-radiation environments, like under laboratory conditions or during overcast days, may underestimate impacts.

As highlighted in earlier studies, water availability or lack thereof is greatly relevant in gauging whether a heat wave will give rise to heat stress (Salvucci & Crafts-Brandner, 2004). If drought prompts a plant to conserve water by lowering stomatal conductance (g_s), it warms up as energy dissipation shifts from latent fluxes (providing cooling) to sensible fluxes (increasing temperatures). Because heat and drought often co-occur naturally (De Boeck *et al.*, 2010), this effect is very relevant in assessing heat wave impacts (Idso, 1982; De Boeck *et al.*, 2016). The potentially misleading nature of T_a in predicting heat stress under varying stomatal conductance is clearly highlighted in our results (Fig. 1, S2, S5-6, see supplementary material section 1).

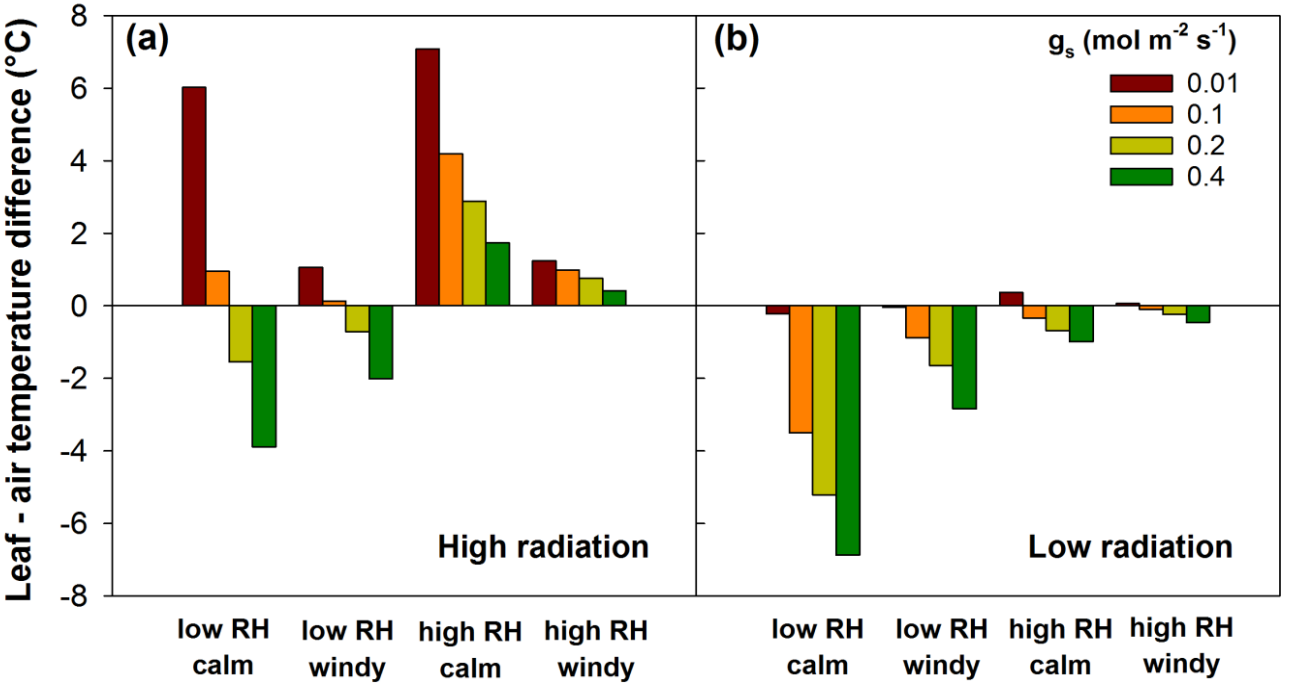


Fig. 1 Modelled leaf-to-air temperature difference depending on type of heat wave and stomatal conductance (g_s). Type of heat wave: high (A) or low (B) incident shortwave radiation (800 or 100 W m^{-2}), high or low relative humidity of the air (RH = 0.90 or 0.45), and calm or windy weather (wind speed 0.1 or 6 m s^{-1}). Air temperature was set to 40 °C in all simulations, and leaf width to 0.005 m.

Whenever other conditions alleviate some amount of heat stress (e.g. less radiation, higher g_s), more wind would counteract such beneficial effects (Fig. 1, S4-5, S7, see supplementary material section 1) through closer coupling between the plant and the air. This may seem counterintuitive as windiness is generally associated with heat dissipation, but the same process also works in the opposite case: when other environmental conditions would exacerbate heat stress, more wind reduces the increase of leaf temperatures. In other words, windy conditions lead to avoidance of the most extreme cases of overheating. Obviously, higher wind speeds promote evapotranspiration, resulting in faster depletion of soil water reserves. This could subsequently lead to lower g_s and thus indirectly promote overheating. As wind speeds in laboratory conditions and/or enclosures are often far below those observed outside (De Boeck *et al.*, 2012), canopy warming may be significantly different from outside as calm conditions tend to exacerbate other effects (Fig. 1, S4-5, S7, see supplementary material section 1).

Also for relative air humidity, the results are counterintuitive, with higher humidity more likely to give rise to heat stress (Fig. 1, S2, S6-7, see supplementary material section 1). Humid conditions are typically regarded as favourable with respect to plant survival since they limit water loss. Heat stress with higher humidity is caused by slower heat dissipation via transpiration as the water vapour gradient between leaf and air is smaller than in the case of drier air. In fact, the combination of low stomatal conductance and high air humidity causes the greatest warming of leaves above the air temperature (Fig. 1). A five-day period featuring air temperatures at vegetation height exceeding 30 °C every day provided us with an opportunity to test whether increasing air humidity diminishes the cooling capacity of leaves. We indeed found a significant relationship between RH and $T_l - T_a$ (Fig. 2), with ± 0.84 °C change per 0.1 increase in RH (excluding the dry day). This is comparable to the slope (0.72 °C per 0.1 increase in RH) found with a model run using conditions similar to the heat wave period (Fig. S8, see supplementary material section 1). Leaf cooling seemed to be reduced on the only day during which irrigation was withheld (Fig. 2): leaves were warmer than the air 32% of the time on the dry day vs. 4% on days with irrigation (even though incident radiation was c. 15% lower on the dry day, while wind speed was similar). We attribute this relative warming to stomatal closure (leaf wilting observed) resulting from drying of the top soil, and subsequent lower transpiration.

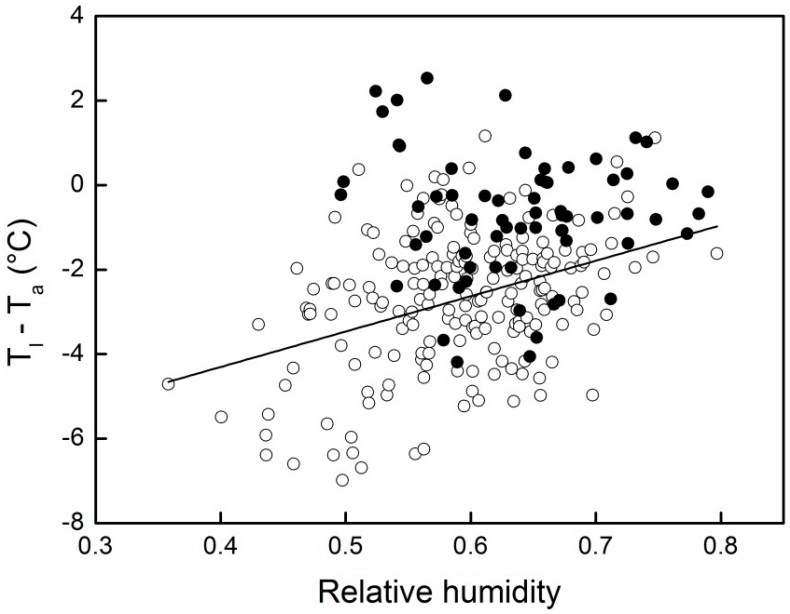


Fig. 2 Differences between leaf (T_l) and air (T_a) temperature in function of relative air humidity (RH) measured on a homogeneous grass stand during 5 heat wave days (1-5 July 2015, Belgium). The grass was irrigated daily (white circles), with the exception of one day (black circles). The linear regression (white data points only) was significant at $p < 0.001$ ($R^2 = 0.13$). The difference between regressions (white vs. black) was significant (ANCOVA, $F_{1,257} = 10.3$; $p = 0.001$, Graphpad Prism). In contrast to the model runs, which focus on one peak air temperature (40 °C) to obtain clean comparisons between differing conditions, the relationship presented here contains more scatter because of factors varying throughout the day such as air temperature, incident radiation, stomatal conductance and wind speed.

The aforementioned trends were observed both for simulations using narrow (Fig. 1) and also for simulations using bigger leaves (Fig. S9, see supplementary material section 1). Any variable increasing the heat load (high radiation) or decreasing heat dissipation (high RH, low wind and g_s) led to higher temperature increases in big compared to in small leaves, however. This is no surprise as larger surfaces result in increased decoupling from air temperatures, which can lead to extreme temperature deviations. In cushion plants, which physically act as a giant leaf, increases of tissue temperatures of 20 °C and more above the air temperature have been

observed (Gauslaa, 1984), illustrating the importance of physical dimensions in energy balances.

Calculations of leaf temperatures are possible at well-equipped sites applying a model such as the one used here. However, increasing quality and decreasing costs of infrared imaging also enable direct quantification of leaf temperatures and variability thereof. Infrared cameras allow the user to select those pixels or zones deemed most appropriate (e.g. excluding bare soil, focusing only on fully developed leaves), improving control and versatility. Automated measurements and batch image processing can render the entire process more efficient, and allow for a high temporal resolution with limited workload. Moreover, simultaneous measurements of incoming shortwave radiation enable data filtering (e.g. clear sky, completely overcast), further improving possibilities during data analysis. More technical background information on extrapolation from leaves to canopies, dealing with temperature variability, improving temperature accuracy and automated image recognition can be found in Jones *et al.* (2009), Jones & Vaughan (2010) and Wang *et al.* (2010).

In conclusion, we clearly demonstrated that exceedance of critical temperatures in plants depends on more variables than air temperature alone. Radiation, wind speed and relative humidity all affect tissue temperatures, depending on plant water status. This implies that heat waves characterized by the same extreme air temperatures may cause little plant damage under some conditions, but could be detrimental to plant growth and survival in other cases. Although heat stress also depends on other factors, like hardening (Neuner & Buchner, 2012) and development stage (Fischer, 2011), the results from this study can help predict when the probability of heat stress occurring is most likely, and can stimulate ecologists and agronomists to shift the focus beyond merely air temperatures when considering heat waves.

2.5 ACKNOWLEDGEMENTS

H. Van De Velde was supported by FWO Vlaanderen. We thank F. Kockelbergh for technical assistance and the referees for valuable suggestions.

2.6 SUPPLEMENTARY MATERIAL

2.6.1 Section 1: supplementary figures

Fig. S1 Homogeneous grass stand equipped with two pyranometers (upward and downward, for each hemisphere), a non-contact infrared thermometer for canopy temperature and a combined air temperature and relative air humidity sensor shielded by wooden panel.

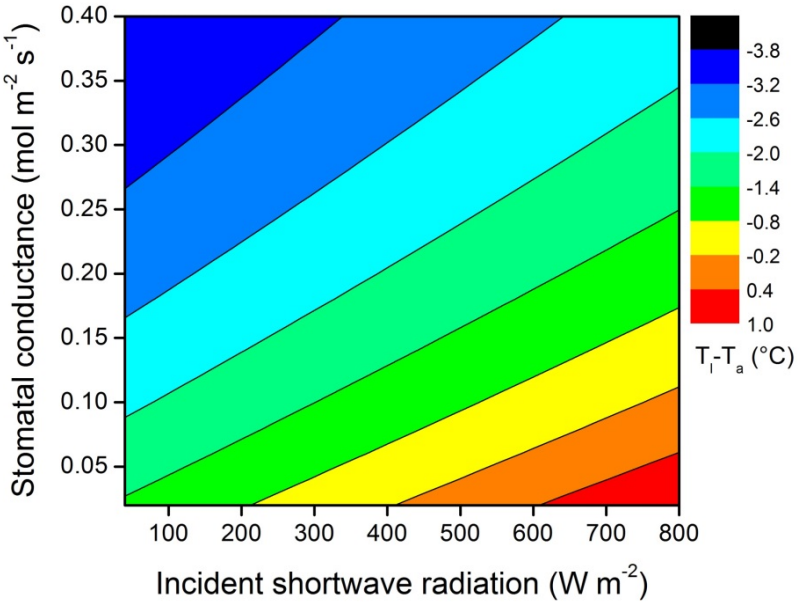


Fig. S2 The influence of incident shortwave radiation and stomatal conductance on the difference between leaf (T_l) and air (T_a) temperatures (depicted by different colours). Generally, more radiation leads to relatively warmer leaves, as does lower stomatal conductance. When stomatal conductance is low, effects of radiation on the leaf – air temperature difference are exacerbated. Other variables were kept constant: air temperature = 40 °C, wind speed = 1.5 m s^{-1}, relative air humidity = 0.6 and leaf diameter = 0.005 m.

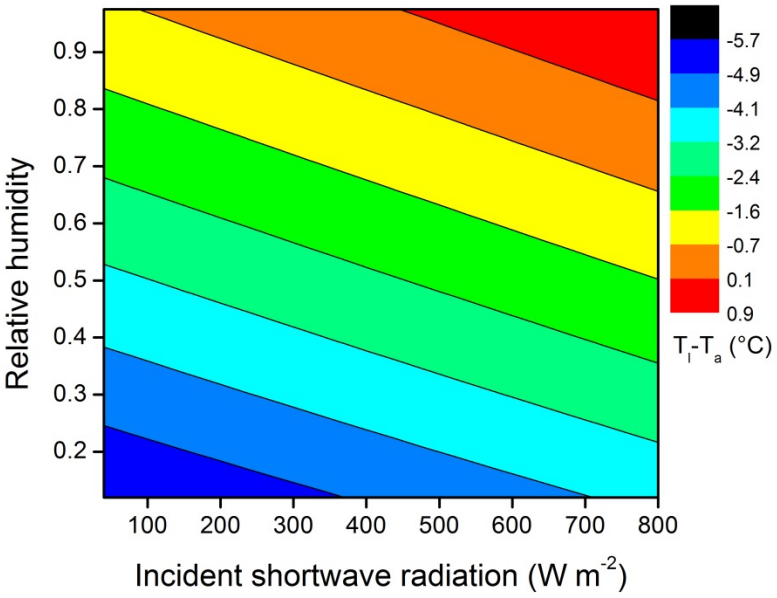


Fig. S3 The influence of incident shortwave radiation and relative air humidity on the difference between leaf (T_l) and air (T_a) temperatures (depicted by different colours). Generally, more radiation leads to relatively warmer leaves, as does higher air humidity. Radiation effects are equivalent at higher and lower air humidity. Other variables were kept constant: air temperature = 40 °C, wind speed = 1.5 m s^{-1}, stomatal conductance = 0.2 mol m^{-2} s^{-1} and leaf diameter = 0.005 m.

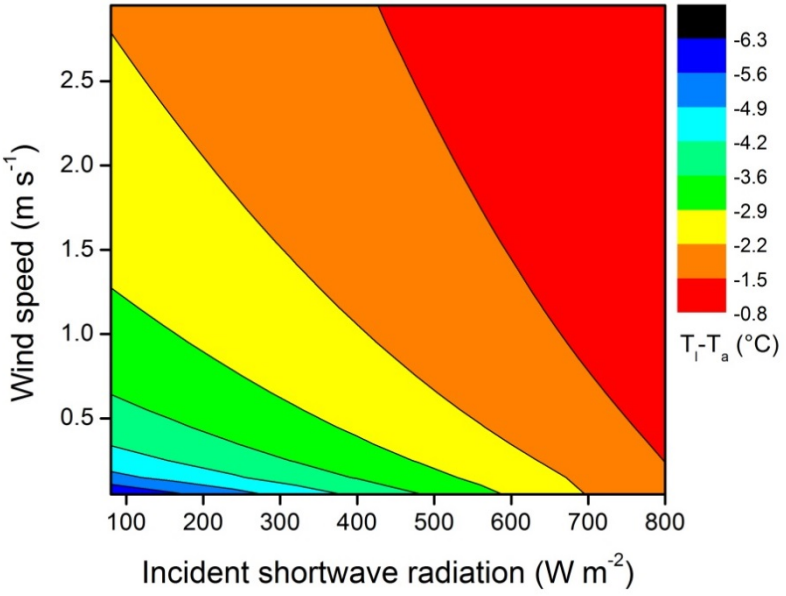


Fig. S4 The influence of incident shortwave radiation and wind speed on the difference between leaf (T_l) and air (T_a) temperatures (depicted by different colours). Generally, more radiation leads to relatively warmer leaves. When wind speed is low, effects of radiation on the leaf – air temperature difference are exacerbated. Other variables were kept constant: air temperature = 40 °C, stomatal conductance = 0.2 mol m^{-2} s^{-1}, relative air humidity = 0.6 and leaf diameter = 0.005 m.

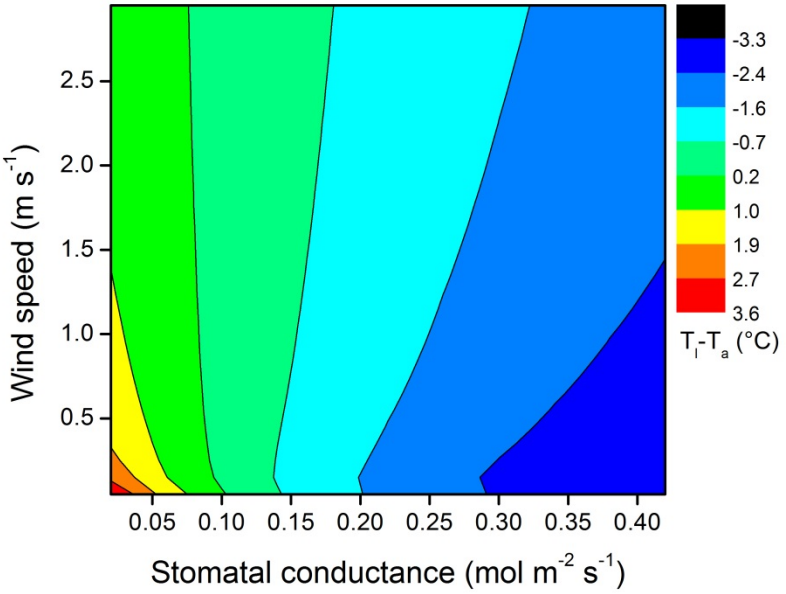


Fig. S5 The influence of stomatal conductance and wind speed on the difference between leaf (T_l) and air (T_a) temperatures (depicted by different colours). Generally, lower stomatal conductance leads to relatively warmer leaves. Low wind speed exacerbates effects of stomatal conductance, while high wind speed dampens these. Other variables were kept constant: air temperature = 40 °C, incident shortwave radiation = 800 W m^{-2}, relative air humidity = 0.6 and leaf diameter = 0.005 m.

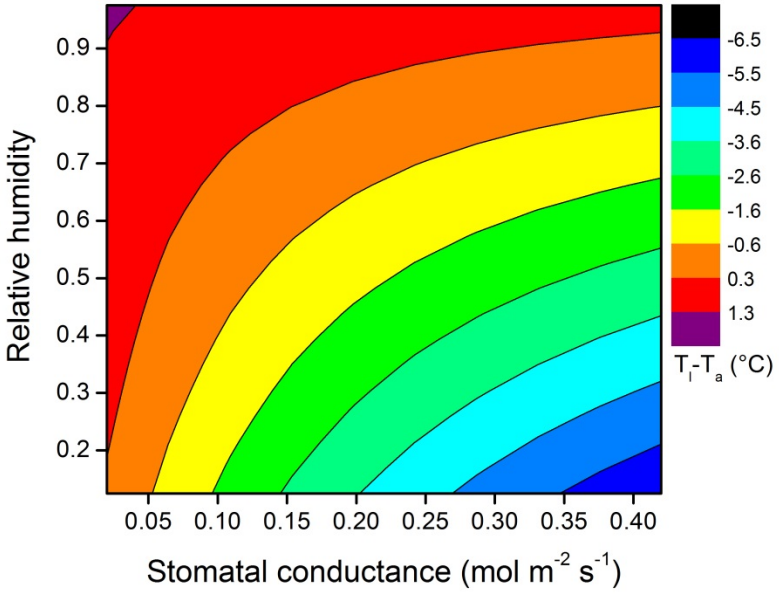


Fig. S6 The influence of stomatal conductance and relative air humidity on the difference between leaf (T_l) and air (T_a) temperatures (depicted by different colours). Generally, lower stomatal conductance leads to relatively warmer leaves, as does higher air humidity. High air humidity dampens the influence of stomatal conductance. Other variables were kept constant: air temperature = 40 °C, wind speed = 1.5 m s^{-1}, incident shortwave radiation = 800 W m^{-2} and leaf diameter = 0.005 m.

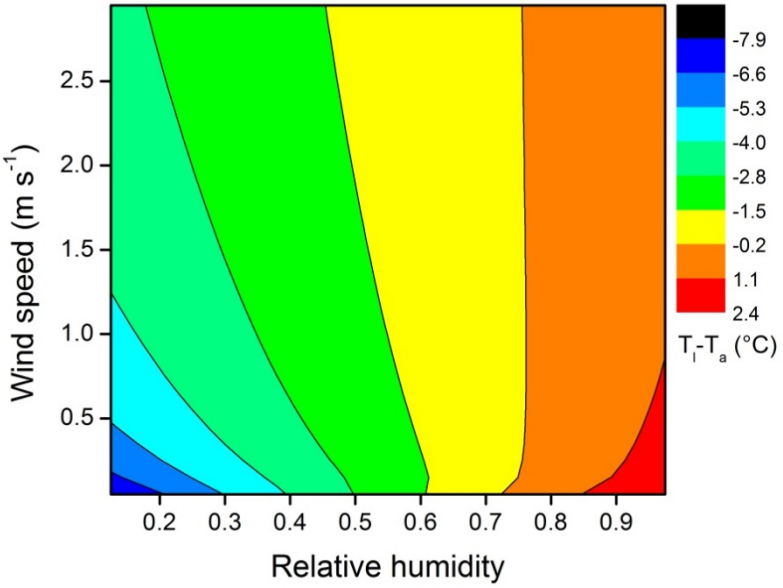


Fig. S7 The influence of relative air humidity and wind speed on the difference between leaf (T_l) and air (T_a) temperatures (depicted by different colours). Generally, higher air humidity leads to relatively warmer leaves. Low wind speeds exacerbate effects of air humidity, while high wind speeds dampen these. Other variables were kept constant: air temperature = 40 °C, stomatal conductance = 0.2 mol m^{-2} s^{-1}, incident shortwave radiation = 800 W m^{-2} and leaf diameter = 0.005 m.

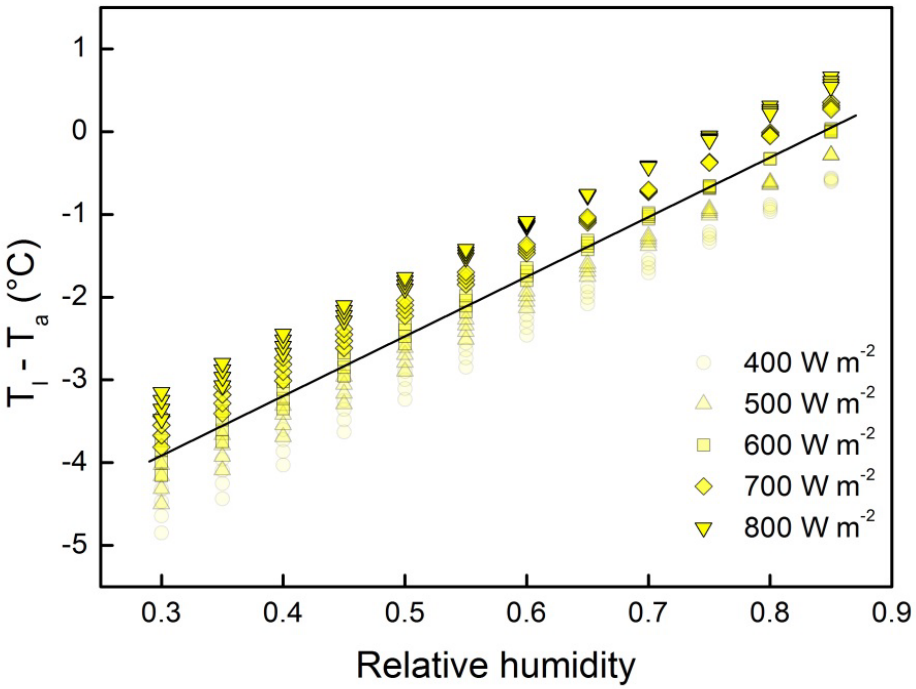


Fig. S8 Modelled influence of relative humidity on leaf (T_l) – air (T_a) temperature differences. Input data reflect conditions similar to those in Fig. 2: T_a = 30 °C, incident shortwave radiation = 400-800 W m^{-2} (marked with different symbols and shades), stomatal conductance = 0.4 mol m^{-2} s^{-1}, wind speed = 0.5-0.8 m s^{-1} (lower wind speed leads to lower T_l-T_a for identical other environmental conditions). Wind speed data at our site were unfortunately not measured during the heat wave due to sensor malfunction and were derived from data of a nearby meteorological station (Lint, Belgium) and correlation (R^2 = 0.80) with data registered on later days (9-23 July).

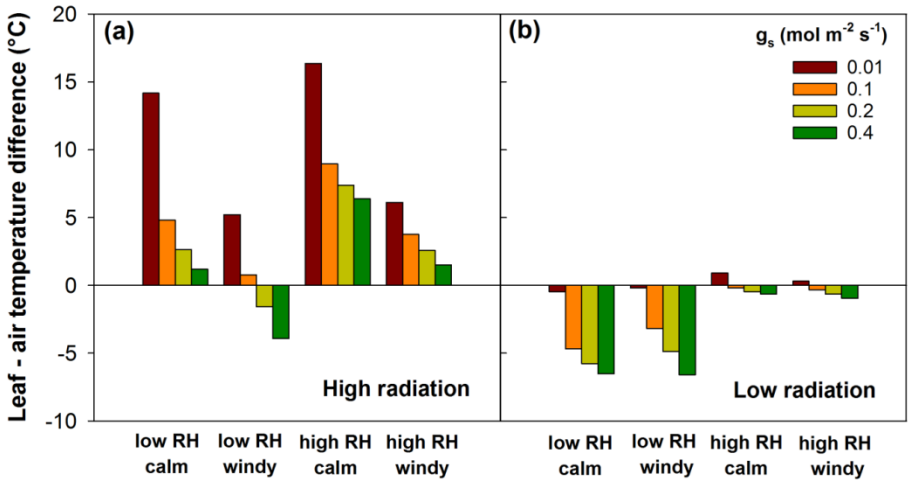


Fig. S9 Modelled leaf-to-air temperature difference depending on type of heat wave and stomatal conductance (g_s). Type of heat wave: high (A) or low (B) incident shortwave radiation (800 or 100 W m^{-2}), high or low relative humidity of the air (RH = 0.90 or 0.45), and calm or windy weather (wind speed 0.1 or 6 m s^{-1}). Air temperature was set to 40 °C in all simulations, and leaf width to 0.2 m.

3 COMBINED ELEVATED CO_2 AND CLIMATE-WARMING INDUCES LAGGED EFFECTS OF DROUGHT IN *LOLIUM PERENNE* AND *PLANTAGO LANCEOLATA*

Adapted from: Van De Velde H, Bonte D, AbdElgawad H, Asard H & Nijs I. (2015) Combined elevated CO_2 and warming induces lagged effects of drought in *Lolium perenne* and *Plantago lanceolata*. Plant ecology, 216, 1047-1059.

3.1 ABSTRACT

Future climate scenarios predict increases in elevated atmospheric CO_2, air temperature and drought, but the impacts of multiple climate change factors on ecosystem functioning remain unclear. In this study, we compared drought responses of plants under future vs. current climate conditions. In addition to focusing on stress during the drought itself, we also examined post-drought lagged effects, and whether warming and elevated CO_2 alter these. We grew monocultures and mixtures of two grassland species (*Lolium perenne* L. and *Plantago lanceolata* L.) in four simulated climate scenarios: (1) current climate, (2) current climate with drought, (3) warmer temperature with drought and (4) combined warming, elevated CO_2 and drought. *L. perenne* and *P. lanceolata* were influenced by the climate scenario but not differently enough to modify the competitive balance. Warming aggravated drought impacts on *L. perenne* and elevated CO_2 only partly compensated for these effects. In a warmer climate, with or without elevated CO_2, drought continued to enhance senescence and mortality in *L. perenne* long after the water shortage, while such lag effects were not observed in current climate. In *P. lanceolata* a similar stimulation of senescence and mortality was induced, but only under combined warming and elevated CO_2. These lag effects induced by the future climate may reduce resilience.

3.2 INTRODUCTION

The global mean air temperature is expected to increase by 2-7 °C by the end of this century as a result of rising levels of atmospheric carbon dioxide (CO_2) and other greenhouse gases (Allison *et al.*, 2009). Increased mean air temperature will likely accompany changes in precipitation, such as prolonged summer drought (IPCC, 2013). Although the effects of future climate on plant growth have been widely explored, most studies have investigated single factor effects such as elevated CO_2, warming, and extreme events, or two-factor combinations of these. Studies that combine all of these climate change components are rare because numerous experimental treatments are usually involved.

Drought stress is one of the major limitations for global plant productivity, primarily through decreased stomatal conductance and down-regulation of photosynthetic machinery (Chaves *et al.*, 2002), including photosynthetic enzyme activity (Reddy *et al.*, 2004) and pigments (Jaleel *et al.*, 2009). Warming can stimulate plant biomass production via higher photosynthesis and/or mineralization rates (Rustad *et al.*, 2001; Wu *et al.*, 2011), but can retard productivity via associated drought stress and heat (De Boeck *et al.*, 2008; Sherry *et al.*, 2008). These associated stresses result from initially enhanced evapotranspiration and soil water depletion (Allen *et al.*, 2003). Similarly, warming is expected to aggravate drought stress. Elevated CO_2 can stimulate plant growth directly through enhanced photosynthesis, or indirectly through reduced water use and higher water-use-efficiency (Morgan *et al.*, 2004), thereby counteracting drought effects (Morgan *et al.*, 2004).

Climate extremes such as drought can cause lag or carry-over effects (Niu *et al.*, 2014). Grassland studies show that dry periods induce reduced productivity which continues long after the dry period (Lauenroth & Sala, 1992; Dunnett *et al.*, 1998; O'Connor *et al.*, 2001). Lagged responses may arise from increased mortality over time (Bigler *et al.*, 2007) and are detectable by comparing plant functioning at the end of the growing season with that just after the drought period. As drought frequency is likely to rise (IPCC, 2013), understanding how quickly plant communities recover from drought under elevated CO_2 and higher temperatures is needed to determine whether insufficient or compromised recovery threatens plant community

stability in future conditions. It is unclear whether or how elevated CO_2 and warming alter lagged plant responses after a drought event.

To address this, we used two common grassland species, *Lolium perenne* L., a perennial grass that forms dense tussocks (Beddows, 1967), and *Plantago lanceolata* L., a rosette-forming perennial forb (Sagar & Harper, 1964). The former has been reported to display isohydric behaviour under drought while *P. lanceolata* is anisohydric (Van den Berge *et al.*, 2014). These differences may determine species-specific biomass responses to changing environmental conditions (Morecroft *et al.*, 2004; Van den Berge *et al.*, 2014). For example, Morgan *et al.* (2011) showed that elevated CO_2 and warming stimulated total aboveground biomass, due to more proportional growth of C_4, but not C_3 grasses. The impact of climate change on species-specific productivity will depends on how climate affects water availability, resource-use-efficiency and availability of growth-limiting resources (Field *et al.*, 1992; De Valpine & Harte, 2001). Ultimately, species-specific productivity responses can alter competitive interactions within plant communities by differentially changing resource requirements among species (Dunnett & Grime, 1999). Consequently, climate change factors could alter plant-plant interactions, but empirical studies so far are rare.

The factors involved in climate change can interact, but there are few studies that document plant response to drought, warming and elevated CO_2 (e.g. Hamerlynck *et al.*, 2000; Dukes *et al.*, 2005; Kongstad *et al.*, 2012; Naudts *et al.*, 2013). Multifactor experiments have shown that combined responses can be smaller than expected from additive, single-factor effects (Wu *et al.*, 2011), consequently multi-factor experiments are needed. Here we investigate effects of a summer drought on grassland monocultures and mixtures, and explore how these effects are modified by warming and elevated CO_2. Apart from focusing on impacts during the water-free period, we also examined whether drought triggers lagged effects over growing season after the event has past, and whether warming and elevated CO_2 alter these. The experimental design consisted of four simulated climate scenarios: (i) current climate, (ii) current climate with drought, (iii) warmer climate with drought and (iv) warmer climate with elevated CO_2 and drought. We hypothesized that (1) warming exacerbates leaf-level drought stress by decreasing the maximal photochemical efficiency and increasing dead biomass, (2) elevated CO_2 mitigates negative warming effects on the

leaf-level drought stress by increasing photochemical efficiency and reducing biomass loss, (3) warming and elevated CO_2 alter the lagged plant response after drought (4) future climate conditions would alter plant-plant interactions.

3.3 MATERIALS AND METHODS

3.3.1 Experimental set-up

The study was conducted at the Drie Eiken Campus, University of Antwerp, Wilrijk, Belgium (51° 09' N, 04° 24'E). The climate experiments took place in 16 south-facing climate-controlled chambers. Details regarding this experimental platform are in Naudts *et al.* (2011). Four climate scenarios (four chambers per scenario) were simulated in an additive design: (1) current temperature and atmospheric CO_2 concentration (current climate, C); (2) current climate including a drought period (D); (3) future temperature and current atmospheric CO_2, including a drought period (DT); and (4) future temperature and atmospheric CO_2, including a drought period ($DTCO_2$).

3.3.2 Microclimate

The current condition chambers (C and D) followed air temperature based on daily averages calculated from the period 1996-2005. Future temperature chambers simulated a continuous 3 °C warming compared to the simulated current climate. Climate scenarios with elevated CO_2 had a target CO_2 concentration of 620 µmol mol^{-1}. Climate manipulations were based on the IPCC-SRES B2-scenario prediction of moderate change for the year 2100 (IPCC, 2001). Air temperature and relative humidity were measured every 0.5 h with a combined humidity–temperature sensor (Siemens QFA66, Erlangen, Germany) and photosynthetically active radiation (PAR) with a quantum sensor (SDEC, type JYP1000, France). CO_2 concentration was measured and regulated with a CO_2 control group with an infrared analyser (WMA-4, PPSystems, Hitchin, UK). During the experiment (DOY 118 – 307, 2010), monthly average air temperature in C and D chambers was 12.3, 16.6, 18.8, 14.7 and 15.5 °C in May, June, July, August and September, respectively. DT and $DTCO_2$ chambers were 3.0 ± 0.8 °C (SD) warmer than current temperature chambers. Average vapour pressure deficit was 0.35 ± 0.02 and 0.46 ± 0.02 kPa (SD) in the climate treatments with ambient and warmed air, respectively. The average daily PAR was 23.1, 25.3, 34.6, 42.1, 39.7 mol m^{-2} d^{-1} in May, June, July, August and September, respectively, and did not differ between chambers (maximum delta of 2.4 ± 0.5 mol m^{-2} d^{-1} (SD), all chambers combined). In the climate scenarios with current CO_2 (C,

D and DT chambers), the concentration was 392 ± 42 µmol mol^{-1} (SD), while it was 615 ± 81 µmol mol^{-1} (SD) in $DTCO_2$.

Water supplied to the chambers was calculated as in Naudts *et al.* (2011). Plants were watered every two days according to the 10 year average of 14 to 15 raining days per month during the growing season. Total monthly irrigation matched 61.5, 64.4, 85.1, 80.2, 80.9 and 69.7 mm in May, June, July, August, September and October, respectively. Water freely drained while capillary rise was prevented by a drainage system placed below the chambers. Profile probe tubes for the PR2 soil moisture sensor (Delta-T Devices Ltd., UK) were installed in four containers, one of each composition (see below). Experimental drought (in D, DT and $DTCO_2$) was attained by withholding water for 20 days (DOY 197-217). The length of the imposed drought was severe but not extreme, based on previous experimental work in the same chambers and soils (Naudts *et al.*, 2011). Soil moisture was measured once a week before the drought (DOY 130-193) and twice a week during the drought period.

3.3.3 Plant communities

Plant communities were established at the end of April (DOY 116-118) by transplanting six-week-old seedlings in PVC containers (19 cm i.dia., 40 cm height), filled with sandy soil (93.2% sand, 4.6% silt, 2.2% clay; field capacity 0.13 $m^3 m^{-3}$; pH 7.6; Kjeldahl-N 0.42 g kg^{-1}; 1% C in humus). We used two common co-occurring species, *L. perenne* and *P. lanceolata*, originating from wild populations in England. Each of the 16 chambers contained six replicates of four plant community compositions: monocultures of *L perenne* and *P. lanceolata*, and mixtures of both species with either *L. perenne* or *P. lanceolata* as the central target plant. Each community contained six individuals planted in a hexagonal grid at 5 cm distance and one individual at the centre of the grid. Mixed communities with *L. perenne* as a central plant contained four individuals of *L. perenne* and three individuals of *P. lanceolata*, and vice versa for *P. lanceolata* central plants. All communities were fertilized with 10 g m^{-2} NH_4NO_3, 5 g m^{-2} P_2O_5, 10 g m^{-2} K_2O and micro-elements (Fe, Mn, Zn, Cu, B, Mo) dissolved in water applied in two days (DOY 140 and 180).

3.3.4 Biomass harvest

The aboveground biomass of one community per composition (so four communities per climate treatment) was harvested in each chamber before and at the end of the drought (DOY 197 and 217), and at the end of the growing season (DOY 307). For each harvest, live and dead biomass was separated by species. All material was dried at 70 °C for 48 h, and then weighed. For statistical analysis, the sum of aboveground biomass per species was divided by the number of that species in each community.

Before the drought, only age-related leaf senescence contributed the dead biomass. After the drought dead biomass resulted from the combination of age-related leaf senescence with leaf and plant mortality induced by drought. As we could not separate these, we will refer to the causes of dead biomass as "senescence and mortality". Furthermore, leaf senescence must be expressed relative to total biomass (Jobbagy & Sala, 2000; Benot *et al.*, 2014). To verify if drought triggers lagged effects on senescence and mortality, we compared the dead fractions of total aboveground biomass between different treatments and times.

3.3.5 Chlorophyll a fluorescence and analysis of photosynthetic pigments

Chlorophyll a fluorescence, which can detect photosynthetic stress effects prior to visible leaf damage (Lichtenthaler & Miehe, 1997), was measured on the youngest fully expanded leaf of each species × composition × chamber combination (2 × 4 × 16). In monocultures, chlorophyll a fluorescence of the target species was measured whereas in mixtures, a measurement was taken from the target species and a heterospecific neighbour species. Measurements were taken in the morning (7-9 h) on 30-min dark-acclimated leaves with a Hansatech Plant Efficiency Analyzer (King's Lynn, Norfolk, UK). Measurements were made on the same day for all treatments. From these the maximum quantum yield of photosystem II was calculated as $F_v/F_m = (F_m-F_0)/F_m$ where F_v = variable fluorescence, F_m = maximum fluorescence and F_0 = steady state fluorescence.

Tissue chlorophyll (Chl) a, Chl b and carotenoids concentrations of the youngest fully expanded leaf of each species × composition × chamber combination (2 × 4 × 16) were determined. Samples of two replicate

communities per composition were taken in each chamber. In monocultures, a measurement was taken from the target species whereas in mixtures, a measurement was taken from the target species and a heterospecific neighbour species. For each measurement, three leaf discs were punched from one leaf per community (base, centre and top) and immediately frozen in liquid nitrogen. Pigments were determined after acetone extraction according to Porra *et al.* (1989) (but see supplementary material section 1). The three subsamples of each leaf were averaged prior to data analysis.

These measurements were performed prior to and after drought (DOY 190 and 218), and at the end of the growing season (DOY 298).

3.3.6 Data analysis

The live and dead aboveground biomass, F_v/F_m, Chl a+b, carotenoids/chlorophyll ratio, carotenoids and the dead fraction of total aboveground biomass of *L. perenne* and *P. lanceolata* were analysed at the three time points during the experiment and separately for *L. perenne* and *P. lanceolata*. To determine the overall effect of the climate scenario (C, D, DT and $DTCO_2$), composition (monoculture or mixture) or their interaction on the measured plant responses, a Permutational Multivariate Analysis of Variance (PERMANOVA; with adonis function in R; (Anderson, 2001)) was performed. This analysis tests to which degree Euclidean distances among and within treatments differ from random expectations. Because it is distribution-free, different measures following different distributions can be integrated into one multivariate analysis. All measured responses were scaled to the maximum in order to give equal weight in the permutational analysis. The attributed chamber was included as a random effect.

General linear mixed models (GLM) in SAS (version 9.2, SAS Institute Inc., Cary, NC) (Littell *et al.*, 1996) were applied to live and dead aboveground biomass, F_v/F_m, F_0 and pigment concentrations with climate scenario and composition as fixed factors. All fluorescence, pigment ratios, and fractions of dead aboveground biomass were arcsine transformed to meet data distribution assumptions. SWC was analysed with repeated measures with DOY, composition and climate scenario as fixed factors. Chamber was included as a random factor nested within climate scenario. Non-significant factors were backwards-excluded from the model. In case of significant effects, *a posteriori* means comparisons using Tukey test corrected for multiple comparisons were made. Effects were considered significant at $P \leq 0.05$.

3.4 RESULTS

3.4.1 Treatment effects

Prior to drought, warming and elevated CO_2 had no effect on the measured plant responses of either species (Table 1). In contrast, after 20 days of drought, climate scenario altered the measured plant responses (Table 1), and these effects were still present after 90 days of recovery. The plant community context (monoculture versus mixture) had only affected *L. perenne* at three time points during the experiment (Table 1). Moreover, the plant composition did not alter target plant responses to any of the climate treatments (Table 1). We will therefore compare the climate treatment effects for all community compositions combined. A summary of the GLM results for the effects of climate scenario, composition and their interaction on the measured plant responses can be found in the supplementary material section 3 (Table S4-S6).

Table 1 PERMANOVA results testing effect of climate and plant composition on plant responses. Plant communities consist of monocultures and mixtures of *Lolium perenne* and *Plantago lanceolata*. Parameters were analysed before the drought (DOY 197), at the end of the drought (DOY 217) and after recovery, at the end of the growing season (DOY 307). P-values are presented in bold when significant (<0.05).

	Lolium perenne			*Plantago lanceolata*		
	Before drought	Drought	Recovery	Before drought	Drought	Recovery
Climate scenario	0.066	**0.001**	**0.006**	0.108	**0.007**	**0.004**
Composition	**0.037**	**0.044**	**0.005**	0.998	0.065	0.638
Climate scenario × composition	0.170	0.585	0.410	0.665	0.817	0.964

3.4.2 Effect of warming and elevated CO_2 on the drought response

3.4.2.1 Drought response under current climate

The drought response under current climate conditions was determined by comparing plant communities in C and D. SWC decreased considerably during the imposed drought from DOY 203 onwards (*a posteriori* comparison, $P < 0.0001$, Fig. 1) and still remained lower after resuming the pre-treatment watering regime (*a posteriori* comparison, $P < 0.0001$, Fig. 1). Drought reduced live aboveground biomass of *L. perenne* by 35% but not that of *P. lanceolata* (Fig. 2; Table 2). In contrast, aboveground dead biomass of *P. lanceolata* was higher in D than in C at the end of the drought, which was not the case for *L. perenne* (Fig. 3; Table 2). *P. lanceolata* dead aboveground biomass remained higher in D than in C after recovery (Fig. 3). By the end of the study, *L. perenne* had nearly recovered from the drought-induced growth reduction (Fig. 2; Table 2). F_v/F_m of both species was not affected by drought, neither at the end of the drought period nor at the end of the season (Fig. 4; Table 2). Chl a+b and carotenoids/chlorophyll ratio of *L. perenne* were not influenced by drought (Fig. 5, S1, see supplementary material section 2; Table 2), while total carotenoid levels increased (Fig. 6; Table 2). *P. lanceolata* leaves had slightly higher Chl a+b, but similar carotenoid levels (Fig. 5, 6; Table 2), leading to decreased carotenoid/chlorophyll ratios in D treatments (Fig. S1, see supplementary material section S1). After recovery, Chl a+b and carotenoids levels of *L. perenne* were similar in D as in C, while *P. lanceolata* leaves had slightly increased carotenoids but similar levels of Chl a+b after recovery (Fig. 5, 6; Table 2).

3.4.2.2 Effect of warming on the drought response

To determine whether warming altered drought responses, we compared D and DT. Warming did not decrease soil water content during the drought period relative to current climate (*a posteriori* comparison, $P = 0.886$, Fig. 1). Nevertheless, *L. perenne* dead aboveground biomass was 56% higher in DT than in D at the end of the drought period and 60% higher after recovery (Fig. 3; Table 2). Concurrently, F_v/F_m of *L. perenne* dropped in DT relative to D by the end of the drought, but increased to pre-drought levels after

recovery (Fig. 4; Table 2). Just after the drought decreased F_v/F_m in *L. perenne* subjected to drought and warming was due to 53% decreased F_m rather than increased F_0 ($F_{3,42}$ = 11.3, P = 0.0049). Chl a+b and carotenoids of *L. perenne* were not affected by the warming at any point during the experiment (Fig. 5, Fig. 6; Table 2). Contrary to *L. perenne*, warming modified none of the responses of *P. lanceolata* to drought, neither at the end of the drought period nor at the end of the growing season (Fig. 2, 3, 4, 5, 6; Table 2), except for carotenoid/chlorophyll ratios which were significantly higher in DT at the end of the drought (Fig. S1, see supplementary material section 2).

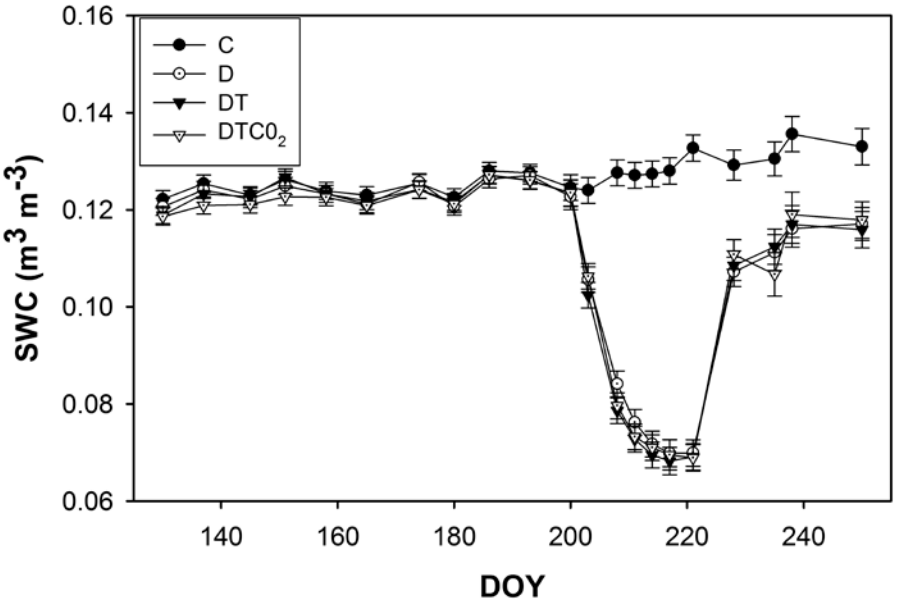


Fig. 1 Time course of soil water content (SWC) in current climate conditions (C, black circle), current climate with drought (D, white circle), warmer climate with drought (DT, black triangle) and warmer climate with elevated CO_2 and drought ($DTCO_2$, white triangle). The drought period was initiated at day of year (DOY) 197 and re-watering started at DOY 217. Means ± SE are indicated (all community compositions combined).

3.4.2.3 Combined effect of warming and elevated CO_2 on the drought response

To test for the effect of elevated CO_2 we compared DT with $DTCO_2$. Ultimately we also compared $DTCO_2$ with D, to know the total influence of a future climate on the drought impact. SWC decreased significantly during the drought period in $DTCO_2$ (*a posteriori* comparison, $P < 0.0001$, Fig. 1), but was not different from DT or D *(a posteriori* comparison, $P = 0.979$ and $P = 0.987$, respectively, Fig. 1). Also the live aboveground biomass of both species in $DTCO_2$ was not different from that of DT or D, at any timepoint during the experiment (Fig. 2; Table 2). A similar response of dead aboveground biomass of *L. perenne* and *P. lanceolata* was apparent between $DTCO_2$ and DT, except for *P. lanceolata*, where dead biomass after recovery in $DTCO_2$ exceeded DT. Dead biomass was always higher in $DTCO_2$ compared to D (Fig. 3; Table 2), except for *P. lanceolata* at the end of drought. For *L. perenne*, elevated CO_2 resulted in higher F_v/F_m (Fig. 4; Table 2), which equalized the stress levels of $DTCO_2$ and D. The same trend can be observed for *P. lanceolata* (Fig. 4). After recovery, the F_v/F_m of both plant species in $DTCO_2$ was not different from that of DT or D (Fig. 4; Table 2). Relative to DT and D treatments, $DTCO_2$ reduced the Chl a+b and carotenoids levels in *L. perenne*, but not in *P. lanceolata* (Fig. 5, 6; Table 2). In the latter, pigment levels in $DTCO_2$ were equal to those under drought in current climate. After recovery, the carotenoid/chlorophyll ratio of both species in $DTCO_2$ did not differ from DT or D plants (Fig. S1, see supplementary material section 2).

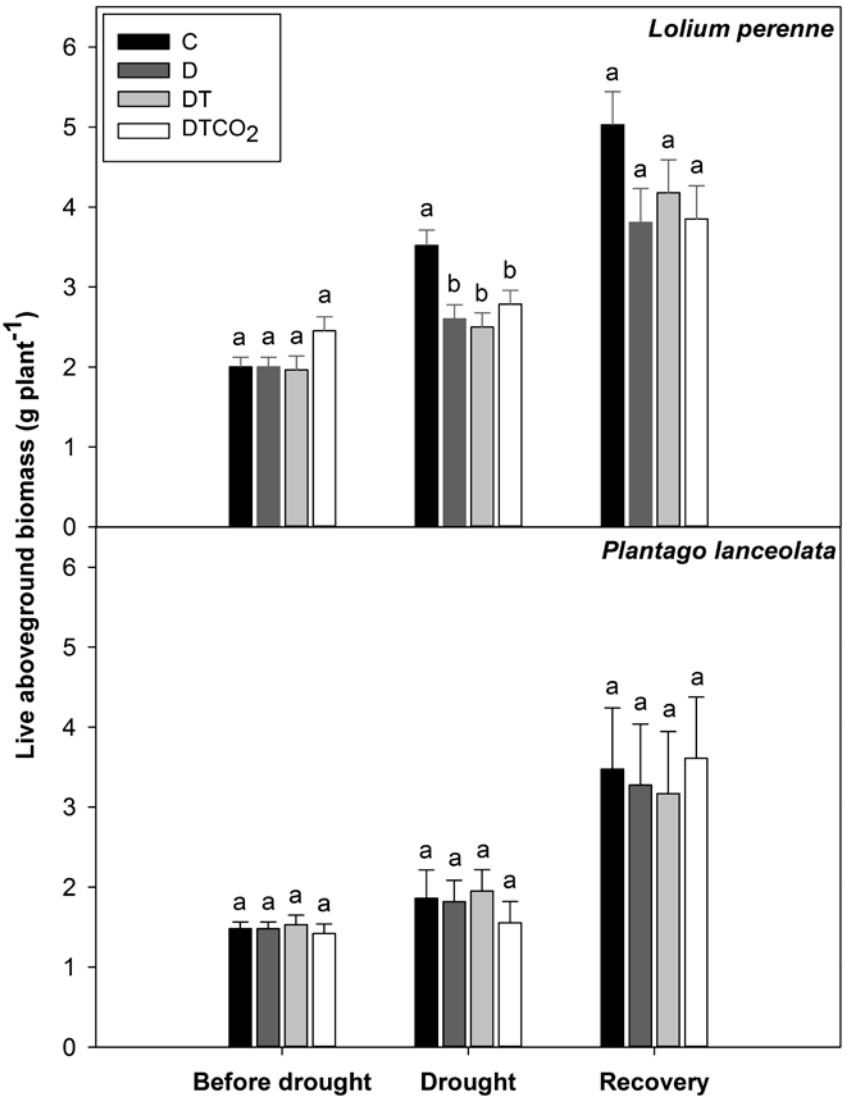


Fig. 2 Live aboveground biomass of *Lolium perenne* (top panel) and *Plantago lanceolata* (bottom panel) before the drought on DOY 197, at the end of the drought on DOY 217 and at the end of the growing season on DOY 307, after recovery. Plants were grown in current climate conditions (C, black bars), current climate with drought (D, dark grey bars), warmer climate with drought (DT, light grey bars) and warmer climate with elevated CO_2 and drought ($DTCO_2$, white bars). The drought period lasted 20 days (DOY 197-217). Means ± SE are indicated (all community compositions combined). Letters indicate differences for posterior comparisons between climate treatments, separately tested for each plant species.

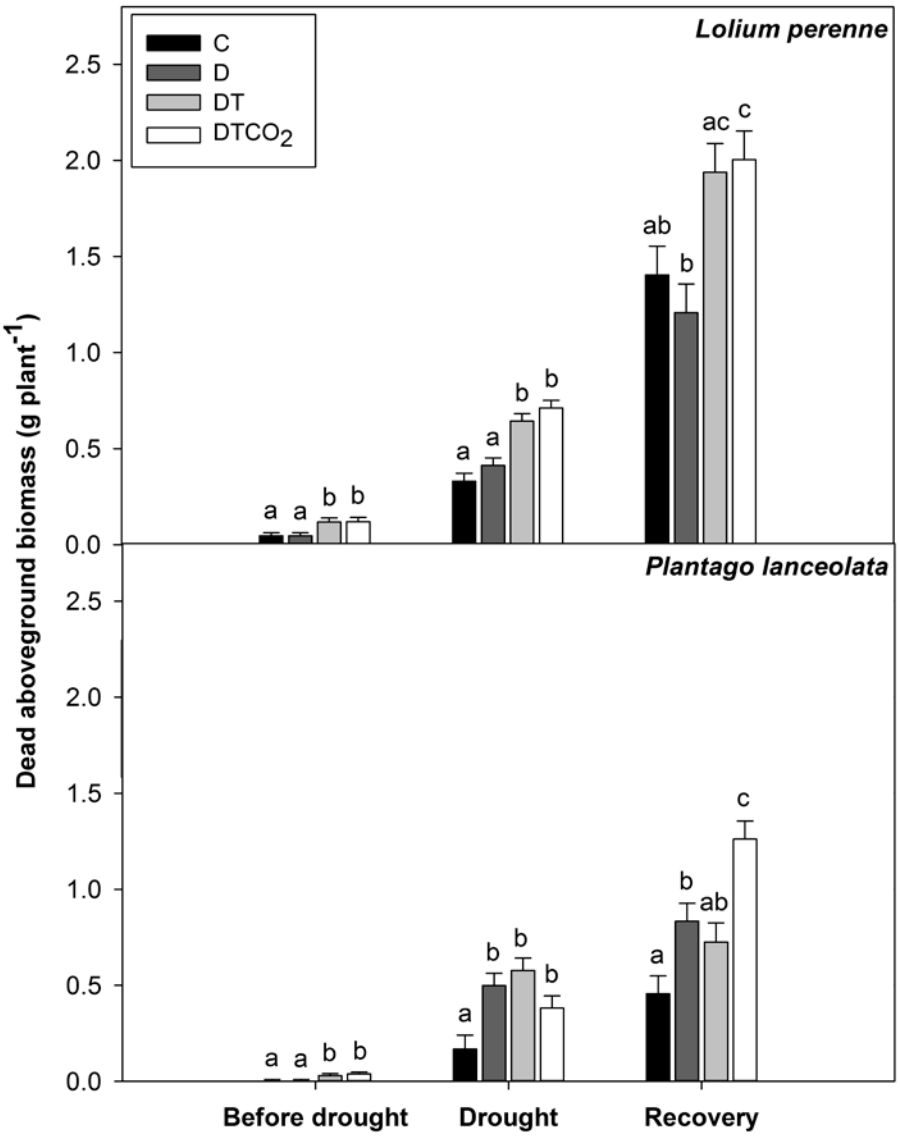


Fig. 3 Dead aboveground biomass of *Lolium perenne* (top panel) and *Plantago lanceolata* (bottom panel) for all community compositions before the drought (DOY 197), at the end of the drought (DOY 217) and end of the growing season (DOY 307). Climate scenarios are as in Fig. 2. Means ± SE are indicated (all community compositions combined). Letters indicate differences for posterior comparisons between climate treatments, separately tested for each plant species.

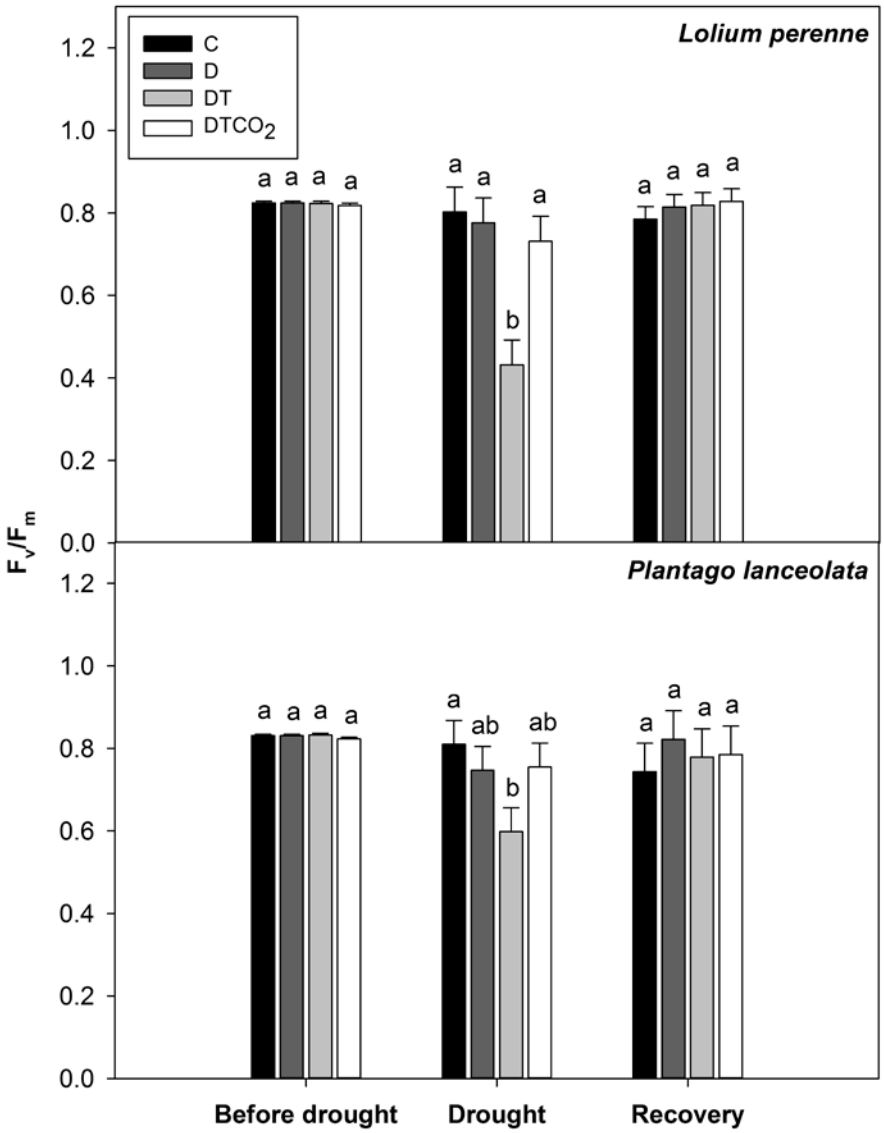


Fig. 4 F_v/F_m of young fully expanded leaves of *Lolium perenne* (top panel) and *Plantago lanceolata* (bottom panel) before drought (DOY 197), end of drought (DOY 217) and at the end of the growing season (DOY 307). Each bar is the mean ± SE, of all community compositions combined. See Fig. 2 for climate scenarios. Letters indicate differences for posterior comparisons between climate treatments.

Table 2 Significance levels (p-values) from *a posteriori* comparisons of plant responses under current climate, current climate with drought, warmer climate with drought and warmer climate with elevated CO_2 and drought. Drought effect was determined by comparing current climate conditions with and without drought, the warming effect by comparing of current climate and warmer climate conditions with drought and the combined warming and elevated CO_2 effect on drought-treated plants by comparing of current climate with drought with those with combined warming, elevated CO_2 and drought. The plant communities consist of monocultures and mixtures of *Lolium perenne* and *Plantago lanceolata.* P-values were corrected for multiple comparisons with Tukey honest significant difference tests, bold results are significant (<0.05). Results obtained before drought (DOY 197), end of the drought (DOY 217) and end of growing season data (DOY 307).

	Drought effect		Effect of warming			Combined effects of warming and elevated CO_2		
	Drought	Recovery	Before drought	Drought	Recovery	Before drought	Drought	Recovery
Lolium perenne								
Live aboveground biomass	**0.006**	0.219	0.983	0.976	0.923	0.099	0.883	0.999
Dead aboveground biomass	0.488	0.789	**0.048**	**0.002**	**0.009**	**0.041**	**<0.001**	**0.004**
F_v/F_m	0.958	0.665	0.968	**<0.001**	0.998	0.622	0.854	0.945
Chl a+b	0.134	0.997	0.917	0.822	0.910	0.988	**0.025**	0.912
Carotenoids	**0.033**	0.637	0.996	0.849	0.941	0.999	**0.004**	0.912

	Drought effect		Effect of warming			Combined effects of warming and elevated CO_2		
	Drought	Recovery	Before drought	Drought	Recovery	Before drought	Drought	Recovery
Plantago lanceolata								
Live aboveground biomass	0.999	0.998	0.940	0.985	0.999	0.916	0.899	0.989
Dead aboveground biomass	**0.001**	**0.037**	**0.001**	0.845	0.854	**<0.001**	0.740	**0.015**
F_v/F_m	0.610	0.566	0.961	0.080	0.876	0.320	0.999	0.917
Chl a+b	**0.046**	0.243	0.999	0.098	0.994	0.924	0.166	0.991
Carotenoids	0.723	**0.035**	0.903	0.899	0.900	0.947	0.402	0.828

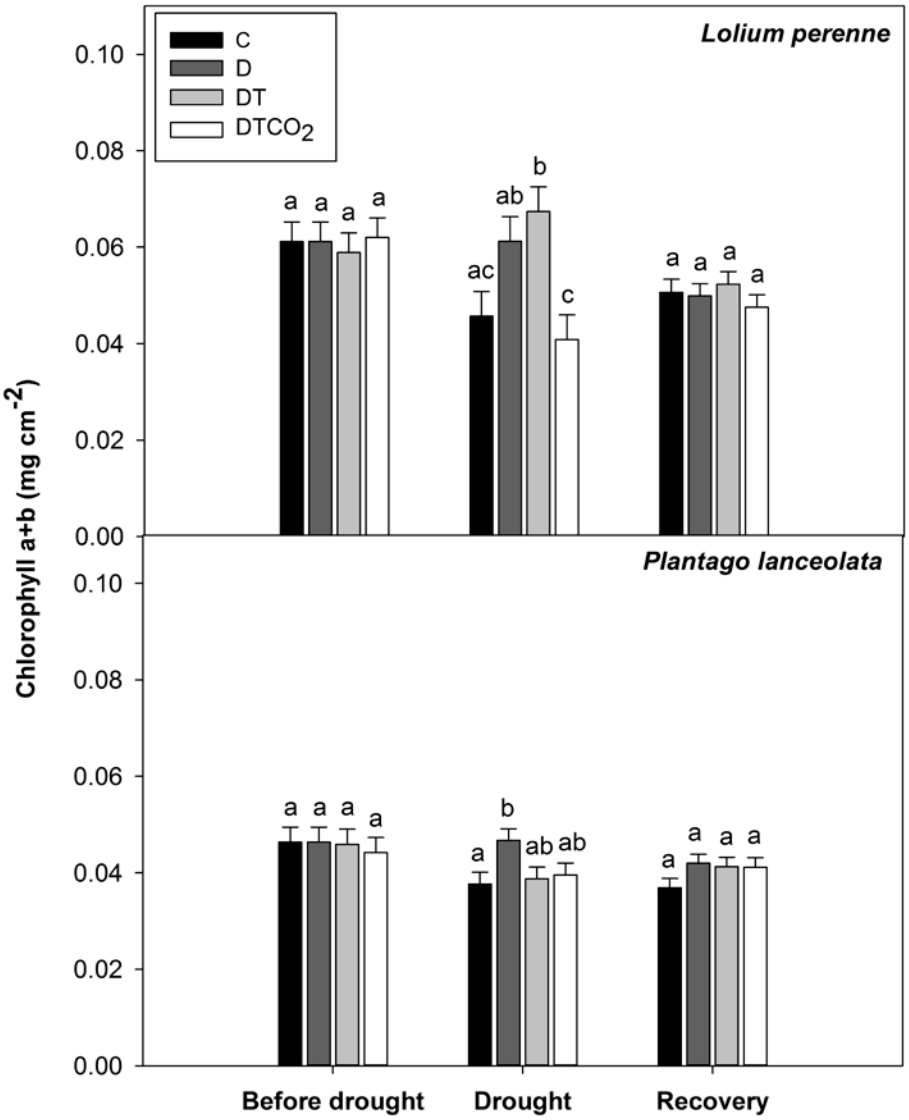


Fig. 5 Leaf chlorophyll a+b of *Lolium perenne* (top panel) and *Plantago lanceolata* (bottom panel) before the drought (DOY 197), end of drought (DOY 217) and at the end of the growing season (DOY 307). Bars are means ± SE, pooled across all community compositions; see Fig. 2 for climate scenarios. Letters indicate differences for posterior comparisons between climate treatments.

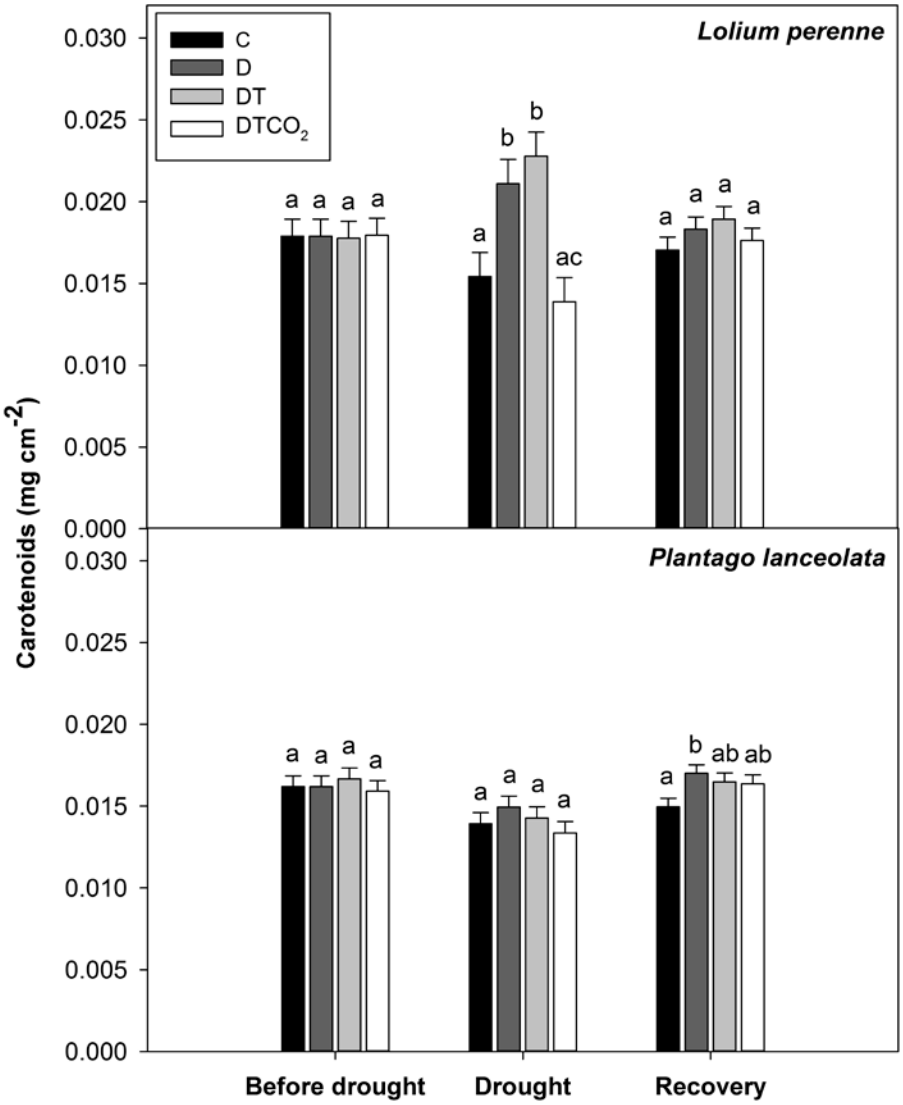


Fig. 6 Leaf carotenoid concentrations of *Lolium perenne* (top panel) and *Plantago lanceolata* (bottom panel) before the drought (DOY 197), end of drought (DOY 217) and at the end of the growing season (DOY 307). Bars are means ± SE, pooled across all community compositions; see Fig. 2 for climate scenarios. Letters indicate differences for posterior comparisons between climate treatments.

3.4.3 Effect of warming and elevated CO_2 on lagged plant responses

To assess lag plant responses of drought we compared the fractions of dead biomass in each climate scenario at the end of the season with those just after the drought. Comparing fractions of dead biomass at the end of the season is not useful as differences between treatments can result from the drought period itself (rather than from a lag effect on senescence) and may still be observable after 90 days of recovery.

In general, climate conditions altered the fraction of dead aboveground biomass in *L. perenne* and *P. lanceolata* ($F_{3,12} = 23.14$, $P < 0.0001$, Fig. 7). Before drought, dead fractions were small and not different between treatments (Fig. 7). By the end of the drought, the dead fraction in *L. perenne* had risen to about 21% in DT and $DTCO_2$, relative to 9% in C (Fig. 7). Without a lag effect of these treatments, this increased dead biomass should reduce through new growth, as pre-drought differences were absent. However, after 90 days of recovery, DT and $DTCO_2$ attained greater fractions of dead biomass, (about 35% in DT and $DTCO_2$ as opposed to 22% in control, Fig. 7), indicating warm temperatures in the drought period induced higher senescence and mortality throughout the remaining growing season. Ambient temperature treatments (C and D) did not differ by the end of the season (Fig. 7). In *P. lanceolata*, the pattern was only slightly different. In this species, drought increased fractional dead aboveground biomass regardless of climate scenario, (about 47% in D and $DTCO_2$ and 50% in DT as opposed to 23% in C, Fig. 7, end of drought data). After recovery, $DTCO_2$ plants largely maintained greater dead biomass fractions relative to controls (56% in $DTCO_2$, 35% in C, Fig. 7), while D and DT plants reached intermediate values (46%, Fig. 7). Fig. 3 showed pre-drought differentiation between the future climates and control. This may imply warming accelerated senescence and mortality. However, the ratio of dead to total biomass showed no difference between future and current treatments (Fig.7, pre-drought data). Moreover, in *P. lanceolata*, the senescence and mortality can be ascribed only to the drought treatment and not to warming because DT did not differ from D just after the drought (Fig. 7). In *L. perenne* just after the drought the dead fraction in DT was significantly higher than in D, so we cannot completely exclude warming itself as a mechanism for enhanced senescence and mortality.

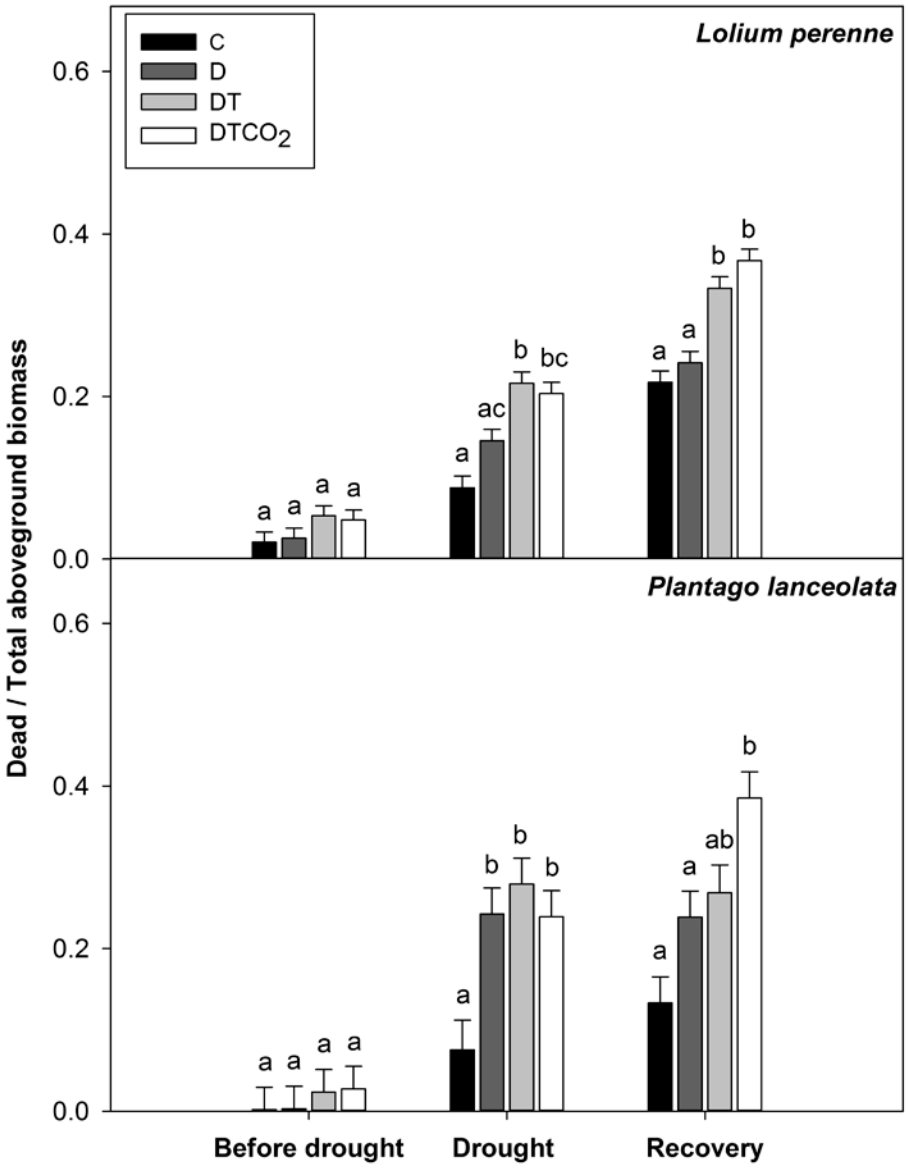


Fig. 7 Dead fraction of total aboveground biomass of *Lolium perenne* (top panel) and *Plantago lanceolata* (bottom panel) before the drought (DOY 197), end of drought (DOY 217) and at the end of the growing season (DOY 307). Bars are means ± SE, pooled across all community compositions; see Fig. 2 for climate scenarios. Letters indicate differences for posterior comparisons between climate treatments.

3.4.4 Effect of drought, warming and elevated CO_2 on plant-plant interactions

The aboveground biomass of *L. perenne* in a mixture with *P. lanceolata* as a central plant was always higher than in the other plant compositions, whereas the aboveground biomass of *P. lanceolata* did not differ between compositions (Table S1-S3, see supplementary material section 3). These plant-plant interaction patterns were not altered by drought, warming or elevated CO_2 at any point over the experiment (Table 1).

3.5 DISCUSSION

Experimental studies that simultaneously vary more than two climate change factors are still rare, and observations are needed to qualify and validate conceptual and theoretical frameworks (Beier *et al.*, 2012). Our results indicate that climate warming exacerbates drought effects on *L. perenne* and that elevated CO_2 only partly compensates for this. Furthermore, drought in a warmer climate with or without elevated CO_2 induced higher senescence and mortality in *L. perenne* long after drought ended, while no such lag effects occurred under current climate. In *P. lanceolata* a similar stimulation of post-drought senescence and mortality occurred with combined warming and elevated CO_2. Notwithstanding these different responses, the imposed climate scenarios did not alter the competitive interactions between these species.

3.5.1 Warming and elevated CO_2 as modifiers of the drought response

L. perenne responded to drought by producing less live biomass, while *P. lanceolata* accumulated more necromass. This may originate from different capacities for water acquisition and transport (Chaves *et al.*, 2002). Van den Berge *et al.* (2014) showed that monocultures of *P. lanceolata* consume more water under drought compared to those of *L. perenne*, owing to higher stomatal conductance at the onset of drought and later stomatal closure. We also observed considerably drier soil conditions in the monocultures of *P. lanceolata*, as opposed to the monocultures of *L. perenne*, irrespectively of the climate scenario (data not shown). The anisohydric behaviour of *P. lanceolata* species matches the absence of significant biomass loss we observed, since prolonged stomatal opening during drought would facilitate CO_2 uptake, while the isohydric strategy of *L. perenne* might explain its reduced growth.

Warming did not modify the live biomass response to drought, suggesting that the plants were equally restrained by the water shortage under current and warmer climate conditions. Contrary to Zavalloni *et al.* (2008), warming also did not enhance soil drying in our communities. This was unexpected because evapotranspiration was anticipated to increase in a warmer climate due to a higher atmospheric demand. However, warming-accelerated

senescence might have contributed to the unaltered soil water availability under warmer conditions (Zavaleta *et al.*, 2003).

Warming did increase dead biomass of *L. perenne* by the end of the drought. At the same time the F_v/F_m decreased in this species. In our study F_v/F_m of *L. perenne* mainly declined by a lower F_m while F_0 remained constant, suggesting reliance on rapidly reversible photoprotection related to enhanced non-photochemical quenching via the xanthophyll cycle (Long *et al.*, 1994). The trend of higher carotenoids levels in DT relative to D also supports this, as xanthophyll carotenoids protect plants from photo-oxidative damage through thermal dissipation (DemmigAdams & Adams, 1996). The complete recovery of the aboveground biomass, F_v/F_m and carotenoids of *L. perenne* at the end of the season indicates photoprotection of photosynthetic tissues, but it should be noted that after the drought period plants developed new leaves, as shown in the increased live biomass at the end of the season.

Elevated CO_2 did not alter the live and dead biomass of either species at the end of the drought, so productivity was equally restrained by water deficit under DT and DTCO_2. Kongstad *et al.* (2012) found that elevated CO_2 did not counterbalance the drought effect on plant growth. Nevertheless, in *L. perenne*, adding CO_2 increased in F_v/F_m and also lowered the concentration of carotenoids compared with DT, indicating that stress levels were alleviated. Likewise, Hamerlynck *et al.* (2000) showed that elevated CO_2 reduced the impact of drought and heat stress on photosynthesis. In our study the direct compensatory effects of elevated CO_2 were too weak to mitigate biomass loss ensuing from drought.

In agreement with our first hypothesis, our results show warming aggravates negative impact of drought in *L. perenne* by reducing PSII photochemical efficiency and inflicting leaf mortality and senescence. Elevated CO_2 seems to compensate for the detrimental effect of warming on drought through increased photochemical protection but not by decreasing the necromass, partly confirming our second hypothesis. Contrary to *L. perenne*, warming or elevated CO_2 did not alter the drought response of *P. lanceolata*.

3.5.2 Effect of warming and elevated CO_2 on lagged plant responses

In *L. perenne* grown in a future climate (DT and $DTCO_2$), the fraction of dead biomass lasted after the drought had ended, while no such lag effect was apparent in current climate conditions by the end of the season. *P. lanceolata* also exhibited post-drought lag effects on the fraction of dead biomass, especially under combined warming and elevated CO_2. Consequently, drought in the current climate did not trigger lagged effects but a future climate induced it, partly confirming our third hypothesis. The persistence of increased dead biomass fractions after drought until the end of the season indicates higher senescence and mortality in a future climate conditions. This cannot be ascribed to incomplete recovery since this would result in lower fractions of dead biomass through new growth. Irrespective of climate scenarios, senescence and mortality became fairly high by the end of the season. Probably, dead biomass accumulation was stimulated by the 90-days recovery period without mowing and greater competition for light during the shortening days in autumn.

Dry years can reduce net primary productivity in following years, relative to predictions based on climate-productivity relationships alone (Lauenroth & Sala, 1992; O'Connor *et al.*, 2001; Wiegand *et al.*, 2004). These lag effects of drought are attributed to various mechanisms. First, carbohydrate reserves under long drought are not replenished, causing mortality (Dunnett *et al.*, 1998). Meristem limitation can also follow after plant, root or tiller mortality after drought (Benson *et al.*, 2004). Changes in stored soil water (Wiegand *et al.*, 2004) and lower nutrient mineralization and organic matter decomposition under drought can drive drought lag effects (Schimel & Parton, 1986). However, in our study, the drought lag effects were observed only under warming treatments. It is noteworthy that adding elevated CO_2 did not alter the fraction of dead leaves compared to DT. The higher senescence and mortality in a future climate (DT and $DTCO_2$) can therefore be ascribed mainly to warming, and elevated CO_2 did not compensate negative warming effects.

In the current study, warming did not enhance soil drying, and SWC of all climate treatments recovered after drought treatment ended, reaching more than 84% of the pre-drought values 10 days after rewatering. Therefore, the

observed lag effect on the fraction of dead biomass in DT is not due to differences in soil moisture. We propose that other mechanisms than those measured, must be at the basis of the observed lag effects. For instance, heat stress may cause cellular damage and secondary stresses, such as osmotic and oxidative stresses (Vinocur & Altman, 2005). Leaf senescence, on the other hand, is controlled by a combination of environmental factors, such as temperature and drought, and endogenous factors including age, reproductive maturity and hormone levels (Munne-Bosch & Alegre, 2004). Environmental factors may affect endogenous factors, accelerating leaf senescence (Munne-Bosch & Alegre, 2004). These mechanisms might explain our finding that the combination of warming and elevated CO_2 maintained drought-induced senescence and mortality long after the drought period.

Theory predicts that abiotic stresses such as drought events reduce the resilience of ecosystems (Scheffer *et al.*, 2001), but experimental studies show that grasslands can recover rapidly (Zavalloni *et al.*, 2008; Walter *et al.*, 2011). While this was generally the case in our study, the lag effects on the fraction of dead biomass induced by drought suggest future climate may reduce resilience. This is especially important as drought is predicted to become more frequent in decades to come (IPCC, 2013), and recurrent extremes have been shown to weaken the resistance of plant assemblages already in current climate, owing to memory effects of previous events (Dreesen *et al.*, 2014).

3.5.3 Effect of drought, warming and elevated CO_2 on plant-plant interactions

The interactions between *L. perenne* and *P. lanceolata* were not influenced by the climate scenario; consequently, we reject our fourth hypothesis. The similar rooting depth of these species (Weeve, 1975) suggests they would compete significantly during drought. However, *L. perenne* is able to suppress the root production of herbaceous species, especially in the top soil (Wardle & Peltzer, 2003), leading to divergent root exploitation zones. Limited interaction between the species owing to root partitioning can therefore not be excluded, and would be in agreement with the observed limited influence of the climatic factors on the neighbour effects. Possibly, more severe droughts are needed for interspecific differences in response to

climate to be expressed, as found in grassland (Grant *et al.*, 2014). We propose further experimental research focussing on the influence of different neighbour species is needed to understand whether and under which circumstances climate change can alter plant-plant interactions.

3.6 ACKNOWLEDGEMENTS

H. Van De Velde is a Research Assistant of the Fund for Scientific Research-Flanders (FWO). We thank N. Calluy and M. Wellens for technical assistance, K. Naudts and J. Van den Berge for field assistance.

3.7 SUPPLEMENTARY MATERIAL

3.7.1 Section 1: material and methods

Pigments were extracted three times in 100% acetone by using a MagNALyser (Roche, Vilvoorde, Belgium; 1 min, 7000 rpm) (Porra *et al.*, 1989). During grinding, a trace of $MgCO_3$ salt was added to neutralize plant acids, as well as Na_2SO_4 to dehydrate the plant tissues. Extract was centrifuged for 15 min at 12000 g, and the supernatant collected. Pigment extract absorbance was determined on a microplate reader (Synergy Mx, Biotek Instruments Inc., Vermont, USA) at λ of 440.5, 644 and 662 nm. Chlorophyll a, b, and total carotenoid concentrations (mg cm^{-2}) were calculated using the equations of Kirk and Allen (1965) and Porra *et al.* (1989) for 100% acetone: Chl a = (11.47 × E664) – (1.93 × E647), Chl b = (20.36 × E647) – (5.5 × E664) and carotenoids = (4.69 × E440.5) – (0.268 × Chl a+b) where E = optimal density (light extinction) at the wavelength indicated. All extractions and measurements were performed in dim light within six hours to avoid pigment decomposition and adsorption on Na_2SO_4.

3.7.2 Section 2: supplementary figure

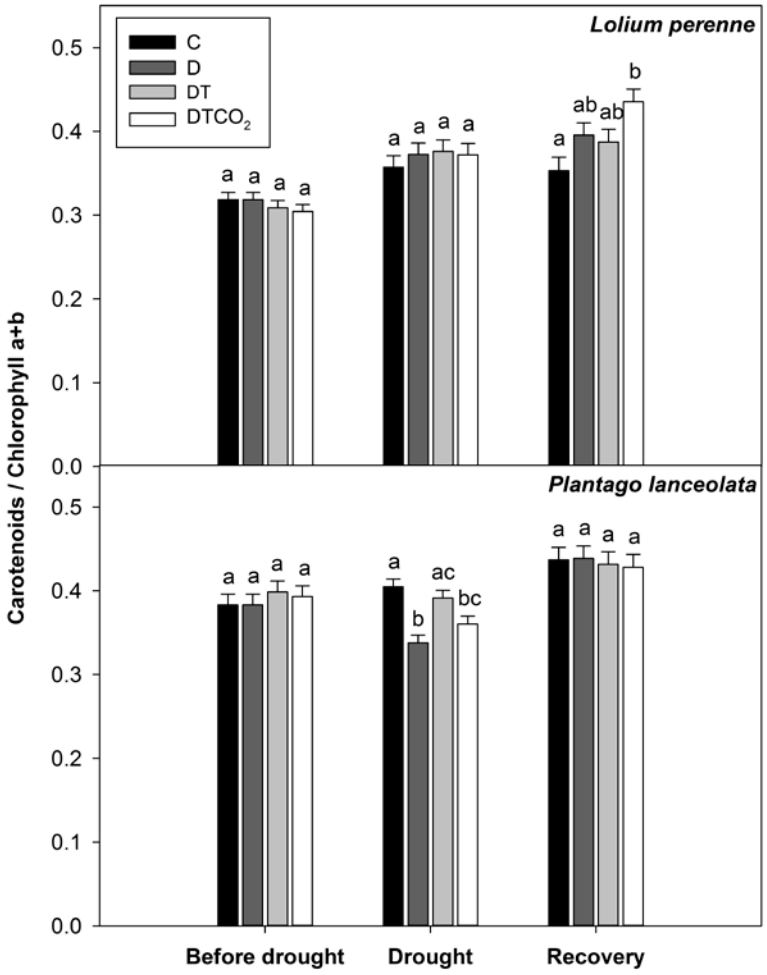


Fig. S1 Carotenoids to chlorophyll ratio in leaves of *Lolium perenne* (top panel) and *Plantago lanceolata* (bottom panel) before the drought on DOY 197, at the end of the drought on DOY 217 and at the end of the growing season on DOY 307, after recovery (means ± SE, all community compositions combined). Plants were grown in current climate conditions (C, black bars), current climate conditions with drought (D, dark grey bars), warmer climate conditions with drought (DT, light grey bars) and future climate conditions with combined warming, elevated CO_2 and drought ($DTCO_2$, white bars). In climate scenarios with a drought period the irrigation was stopped for 20 days (DOY 197-217). Letters indicate differences for posterior comparisons between climate treatments, separately tested for each plant species at the three aforementioned timepoints in the experiment.

3.7.3 Section 3: supplementary tables

Table S1 Effects of plant composition on measured parameters before the drought (DOY 197). The plant communities had four different compositions: (1) monoculture of *Lolium perenne*, (2) monoculture of *Plantago lanceolata*, (3) mixture of both species with *L. perenne* as a central plant (Mixture *L. perenne*) and (4) mixture of both species with *P. lanceolata* as a central plant (Mixture *P. lanceolata*). Values are means ± SE (all climate scenarios combined). Letters indicate differences for posterior comparisons between plant compositions, separately tested for each plant species at three aforementioned moments in the experiment.

	Lolium perenne			*Plantago lanceolata*		
	Monoculture	Mixture *L. perenne*	Mixture *P. lanceolata*	Monoculture	Mixture *L. perenne*	Mixture *P. lanceolata*
Live aboveground biomass (g plant^{-1})	1.96 ± 0.15^{a}	1.90 ± 0.15^{a}	2.57 ± 0.15^{b}	1.62 ± 0.11^{a}	1.51 ± 0.11^{a}	1.31 ± 0.11^{a}
Dead aboveground biomass (g plant^{-1})	0.0961 ± 0.0142^{a}	0.0774 ± 0.0142^{a}	0.112 ± 0.014^{a}	0.0125 ± 0.0033^{a}	0.0312 ± 0.0033^{a}	0.0258 ± 0.0033^{a}
F_v/F_m	0.822 ± 0.005^{a}	0.825 ± 0.005^{a}	0.818 ± 0.005^{a}	0.829 ± 0.004^{a}	0.825 ± 0.004^{a}	0.833 ± 0.004^{a}
Chl a+b (mg cm^{-2})	0.0537 ± 0.0026^{a}	0.0604 ± 0.0032^{ab}	0.0681 ± 0.0032^{b}	0.0445 ± 0.0031^{a}	0.0427 ± 0.0031^{a}	0.0430 ± 0.0031^{a}
Carotenoids (mg cm^{-2})	0.0167 ± 0.0007^{a}	0.0181 ± 0.0009^{a}	0.0188 ± 0.0009^{a}	0.0165 ± 0.0007^{a}	0.0161 ± 0.0007^{a}	0.0161 ± 0.0007^{a}

Table S2 Effects of plant composition on measured parameters at the end of the drought (DOY 217). The plant communities had four different compositions: (1) monoculture of *Lolium perenne*, (2) monoculture of *Plantago lanceolata*, (3) mixture of both species with *L. perenne* as a central plant (Mixture *L. perenne*) and (4) mixture of both species with *P. lanceolata* as a central plant (Mixture *P. lanceolata*). Values are means ± SE (all climate scenarios combined). Letters indicate differences for posterior comparisons between plant compositions, separately tested for each plant species at three aforementioned moments in the experiment.

	Lolium perenne			*Plantago lanceolata*		
	Monoculture	Mixture *L. perenne*	Mixture *P. lanceolata*	Monoculture	Mixture *L. perenne*	Mixture *P. lanceolata*
Live aboveground biomass (g plant^{-1})	2.41 ± 0.15^{a}	2.79 ± 0.16^{a}	3.35 ± 0.15^{b}	2.07 ± 0.25^{a}	1.82 ± 0.25^{a}	1.50 ± 0.24^{a}
Dead aboveground biomass (g plant^{-1})	0.429 ± 0.034^{a}	0.524 ± 0.036^{ab}	0.622 ± 0.034^{b}	0.496± 0.016^{a}	0.299 ± 0.016^{b}	0.373 ± 0.016^{ab}
F_v/F_m	0.760 ± 0.044^{a}	0.606 ± 0.044^{b}	0.656 ± 0.044^{ab}	0.713 ± 0.040^{a}	0.751 ± 0.040^{a}	0.686 ± 0.040^{a}
Chl a+b (mg cm^{-2})	0.0500 ± 0.0036^{a}	0.0578 ± 0.0050^{a}	0.0536 ± 0.0050^{a}	0.0435 ± 0.0020^{a}	0.0429 ± 0.0020^{a}	0.0357 ± 0.0021^{b}
Carotenoids (mg cm^{-2})	0.0176 ± 0.0010^{a}	0.0198 ± 0.0015^{a}	0.0175 ± 0.0015^{a}	0.0149 ± 0.0006^{a}	0.0146 ± 0.0006^{ab}	0.0129 ± 0.0006^{b}

Table S3 Effects of plant composition on measured parameters at the end of the growing season, after recovery (DOY 307). The plant communities had four different compositions: (1) monoculture of *Lolium perenne*, (2) monoculture of *Plantago lanceolata*, (3) mixture of both species with *L. perenne* as a central plant (Mixture *L. perenne*) and (4) mixture of both species with *P. lanceolata* as a central plant (Mixture *P. lanceolata*). Values are means ± SE (all climate scenarios combined). Letters indicate differences for posterior comparisons between plant compositions, separately tested for each plant species at three aforementioned moments in the experiment.

	Lolium perenne			*Plantago lanceolata*		
	Monoculture	Mixture *L. perenne*	Mixture *P. lanceolata*	Monoculture	Mixture *L. perenne*	Mixture *P. lanceolata*
Live aboveground biomass (g plant^{-1})	3.60 ± 0.26^{a}	4.19 ± 0.26^{a}	4.85 ± 0.26^{b}	3.40 ± 0.48^{a}	3.22 ± 0.47^{a}	3.53 ± 0.47^{a}
Dead aboveground biomass (g plant^{-1})	1.19 ± 0.13^{a}	1.60± 0.13^{a}	2.13 ± 0.13^{b}	0.973 ± 0.085^{a}	0.833 ± 0.081^{ab}	0.651 ± 0.081^{b}
F_v/F_m	0.812 ± 0.020^{a}	0.824 ± 0.020^{a}	0.789 ± 0.020^{a}	0.763 ± 0.049^{a}	0.793 ± 0.049^{a}	0.745 ± 0.049^{a}
Chl a+b (mg cm^{-2})	0.0490 ± 0.0019^{a}	0.0519 ± 0.0025^{a}	0.0494 ± 0.0026^{a}	0.0399 ± 0.0017^{a}	0.0394 ± 0.0016^{a}	0.0417 ± 0.0017^{a}
Carotenoids (mg cm^{-2})	0.0176 ± 0.0005^{a}	0.0182 ± 0.0007^{a}	0.0181 ± 0.0008^{a}	0.0161 ± 0.0004^{a}	0.0161 ± 0.0005^{a}	0.0164 ± 0.0005^{a}

Table S4 Summary of GLM results for effects of climate scenario and plant composition on measured plant responses before the drought (DOY 197). The plant communities had four different compositions: (1) monoculture of *Lolium perenne*, (2) monoculture of *Plantago lanceolata*, (3) mixture of both species with *L. perenne* as a central plant and (4) mixture of both species with *P. lanceolata* as a central plant. P-values are presented in bold when significant (≤ 0.05).

Measurement	Treatment	df	F	P
Lolium perenne				
Live aboveground biomass	Climate scenario	2,39	2.57	0.0894
	Composition	2,39	5.07	**0.011**
	Climate scenario × composition	4,39	0.59	0.6705
Dead aboveground biomass	Climate scenario	2,13	5.52	**0.0184**
	Composition	2,26	3.23	0.0558
	Climate scenario × composition	4,26	0.58	0.6828
Fv/Fm	Climate scenario	2,39	0.42	0.6585
	Composition	2,39	0.60	0.5546
	Climate scenario × composition	4,39	0.59	0.6708
Chl a+b	Climate scenario	2,10	0.16	0.8551
	Composition	2,78	10.43	**<0.0001**
	Climate scenario × composition	4,78	3.57	**0.0100**
Carotenoids	Climate scenario	2,10	0.14	0.8698
	Composition	2,78	3.39	**0.0387**
	Climate scenario × composition	4,78	2.50	**0.0491**
Carotenoids/chl a+b	Climate scenario	2,87	0.53	0.5903
	Composition	2,87	5.89	**0.0040**
	Climate scenario × composition	4,87	0.54	0.7098

Measurement	Treatment	df	F	P
Plantago lanceolata				
Live aboveground biomass	Climate scenario	2,39	0.20	0.8228
	Composition	2,39	1.35	0.2707
	Climate scenario × composition	4,39	0.59	0.6747
Dead aboveground biomass	Climate scenario	2,39	6.42	**0.0039**
	Composition	2,39	1.51	0.2343
	Climate scenario × composition	4,39	3.59	**0.0139**
Fv/Fm	Climate scenario	2,39	1.33	0.2770
	Composition	2,39	1.15	0.3277
	Climate scenario × composition	4,39	0.55	0.7007
Chl a+b	Climate scenario	2,62	0.10	0.9072
	Composition	2,62	0.10	0.9041
	Climate scenario × composition	4,62	0.65	0.6323
Carotenoids	Climate scenario	2,62	0.29	0.7486
	Composition	2,62	0.10	0.9050
	Climate scenario × composition	4,62	0.69	0.6001
Carotenoids/chl a+b	Climate scenario	2,62	0.30	0.7440
	Composition	2,62	0.06	0.9421
	Climate scenario × composition	4,62	2.08	0.0947

Table S5 Summary of GLM results for effects of climate scenario and plant composition on measured plant responses at the end of the drought (DOY 217). The plant communities had four different compositions: (1) monoculture of *Lolium perenne*, (2) monoculture of *Plantago lanceolata*, (3) mixture of both species with *L. perenne* as a central plant and (4) mixture of both species with *P. lanceolata* as a central plant. P-values are presented in bold when significant (≤ 0.05).

Measurement	Treatment	df	F	P
Lolium perenne				
Live aboveground biomass	Climate scenario	3,34	4.91	**0.0061**
	Composition	2,34	8.98	**0.0007**
	Climate scenario × composition	6,34	0.52	0.7871
Dead aboveground biomass	Climate scenario	3,23	19.97	**<0.0001**
	Composition	2,23	8.04	**0.0023**
	Climate scenario × composition	6,23	0.99	0.4571
Fv/Fm	Climate scenario	3,36	16.04	**<0.0001**
	Composition	2,36	4.13	**0.0242**
	Climate scenario × composition	6,36	2.30	0.0552
Chl a+b	Climate scenario	3,116	5.34	**0.0018**
	Composition	2,116	0.79	0.4551
	Climate scenario × composition	6,116	0.32	0.9270
Carotenoids	Climate scenario	3,116	7.92	**<0.0001**
	Composition	2,116	0.83	0.4398
	Climate scenario × composition	6,116	0.74	0.6195
Carotenoids/chl a+b	Climate scenario	3,116	0.30	0.8231
	Composition	2,116	3.77	**0.0260**
	Climate scenario × composition	6,116	0.17	0.9839

Measurement	Treatment	df	F	P
Plantago lanceolata				
Live aboveground biomass	Climate scenario	3,31	0.37	0.7754
	Composition	2,31	1.23	0.3073
	Climate scenario × composition	6,31	0.42	0.8618
Dead aboveground biomass	Climate scenario	3,22	7.87	**0.0009**
	Composition	2,22	3.33	0.0547
	Climate scenario × composition	6,22	1.10	0.3928
Fv/Fm	Climate scenario	3,36	4.84	**0.0063**
	Composition	2,36	0.64	0.5336
	Climate scenario × composition	6,36	0.92	0.4888
Chl a+b	Climate scenario	3,83	2.94	**0.0378**
	Composition	2.83	4.12	**0.0196**
	Climate scenario × composition	6,83	1.41	0.2208
Carotenoids	Climate scenario	3,11	0.84	0.4989
	Composition	2,70	3.22	**0.0459**
	Climate scenario × composition	6,70	0.53	0.7832
Carotenoids/chl a+b	Climate scenario	3,83	9.51	**<0.0001**
	Composition	2,83	1.58	0.2114
	Climate scenario × composition	6,83	2.93	**0.0122**

Table S6 Summary of GLM results for effects of climate scenario and plant composition on measured plant responses after recovery (DOY 307). The plant communities had four different compositions: (1) monoculture of *Lolium perenne*, (2) monoculture of *Plantago lanceolata*, (3) mixture of both species with *L. perenne* as a central plant and (4) mixture of both species with *P. lanceolata* as a central plant. P-values are presented in bold when significant (≤ 0.05).

Measurement	Treatment	df	F	P
Lolium perenne				
Live aboveground biomass	Climate scenario	3,12	1.79	0.2026
	Composition	2,23	10.78	**0.0005**
	Climate scenario × composition	6,23	0.98	0.4596
Dead aboveground biomass	Climate scenario	3,24	7.94	**0.0008**
	Composition	2,24	15.10	**<0.0001**
	Climate scenario × composition	6,24	1.74	0.1551
Fv/Fm	Climate scenario	3,36	1.07	0.3757
	Composition	2,36	0.68	0.5109
	Climate scenario × composition	6,36	0.98	0.4536
Chl a+b	Climate scenario	3,102	1.45	0.2328
	Composition	2,102	0.55	0.5781
	Climate scenario × composition	6,102	2.18	0.0513
Carotenoids	Climate scenario	3,102	0.92	0.4344
	Composition	2,102	0.44	0.6449
	Climate scenario × composition	6,102	0.92	0.4807
Carotenoids/chl a+b	Climate scenario	3,102	4.07	**0.0089**
	Composition	2,102	2.60	0.0789
	Climate scenario × composition	6,102	2.48	**0.0276**

Measurement	Treatment	df	F	P
Plantago lanceolata				
Live aboveground biomass	Climate scenario	3,12	0.06	0.9822
	Composition	2,23	0.21	0.8125
	Climate scenario × composition	6,23	0.64	0.6942
Dead aboveground biomass	Climate scenario	3,23	14.61	**<0.0001**
	Composition	2,23	4.81	**0.0180**
	Climate scenario × composition	6,23	1.75	0.1542
Fv/Fm	Climate scenario	3,36	0.55	0.6520
	Composition	2,36	0.18	0.8386
	Climate scenario × composition	6,36	0.72	0.6324
Chl a+b	Climate scenario	3,73	1.41	0.2474
	Composition	2,73	0.55	0.5818
	Climate scenario × composition	6,73	0.32	0.9259
Carotenoids	Climate scenario	3,73	2.60	**0.0492**
	Composition	2,73	0.20	0.8203
	Climate scenario × composition	6,73	0.15	0.9887
Carotenoids/chl a+b	Climate scenario	3,12	0.07	0.9733
	Composition	2,62	3.01	0.0564
	Climate scenario × composition	6,62	0.72	0.6337

4 WARMING AFFECTS DIFFERENT COMPONENTS OF PLANT-HERBIVORE INTERACTION IN A SIMPLIFIED COMMUNITY BUT NOT NET INTERACTION STRENGTH

Adapted from: Van De Velde H, Nijs I & Bonte D. (2017) Warming affects different components of plant-herbivore interaction in a simplified community but not net interaction strength. Oikos, 126(2), 285-295.

4.1 ABSTRACT

Global warming impacts natural communities through effects on performance of individual species and through changes in the strength of interactions between them. While there is a body of evidence of the former, we lack experimental evidence on potential changes in interaction strengths. Knowledge about multispecies interactions is fundamental to understand the regulation of biodiversity and the impact of climate change on communities. This study investigated the effect of warming on a simplified community consisting of three species: rosy apple aphid *Dysaphis plantaginea* feeding on plantain, *Plantago lanceolata*, and a heterospecific neighbouring plant species, perennial ryegrass, *Lolium perenne*. The aphid does not feed on *L. perenne*. The experimental design consisted of monocultures and mixtures of *L. perenne* and *P. lanceolata* at three temperature levels. We did not find indication for indirect temperature effects on *D. plantaginea* through changes in leaf nitrogen or relative water content. However, experimental warming affected the life history traits of the aphid directly, in a non-linear manner. Aphids performed best at moderate warming, where they grew faster and had a shorter generation time. In spite of the increased population growth of the aphids under warming, the herbivory rates were not changed and consequently the plant-herbivore interaction was not altered under warming. This suggests reduced consumption rates at higher temperature. Also plant competition affected the aphids but through an interaction with temperature. We provide proof-of-concept that net interactions between plants and herbivores should not change under warming despite direct effects of warming on herbivores when plant-plant interaction are considered. Our study stresses the importance of indirect non–trophic interactions as an additional layer of complexity to improve our understanding of how trophic interactions will alter under climate change.

4.2 INTRODUCTION

The global mean air temperature is expected to increase as a result of rising levels of atmospheric CO_2 and other greenhouse gases (IPCC, 2014). Numerous studies provide evidence for effects of anthropogenic warming on biota but most of them have concentrated on the level of individuals and species. For example, temperature is the dominant abiotic factor for poikilothermic animals, such as insects, which have limited ability to regulate their internal temperature. Therefore, warming has the potential to affect most life history parameters of terrestrial insects. Studies have revealed that warming shortens development time (Bale *et al.*, 2002) and increases fecundity (Meisner *et al.*, 2014) of insect herbivores until some threshold. Temperature also regulates plant productivity but in a non-linear fashion; warming can stimulate plant biomass production via higher photosynthesis and/or mineralization rates (Rustad *et al.*, 2001; Wu *et al.*, 2011), but retards it via associated drought and heat stress (De Boeck *et al.*, 2008; Sherry *et al.*, 2008). While such influences of warming at the single species level are fairly well understood, in nature species are connected in complex networks, therefore interactions such as competition and herbivory need to be considered.

The effect of temperature on life history processes (e.g. development, growth, reproduction, mortality) can be described by the thermal response curve, usually an asymmetric parabola (Logan *et al.*, 1976; Huey & Kingsolver, 1989). The curves may differ between species due to different levels of performance of the response, different rates of response or different peak or optimal temperatures (Dell *et al.*, 2014). Such asymmetries in the thermal responses of interacting species can subsequently induce qualitative and quantitative changes in consumer-resource dynamics, with important consequences for the dynamics and persistence of populations and communities (Dell *et al.*, 2014). For instance, the growth rate of insect herbivores responds more strongly to temperature than the growth rate of plants (Bale *et al.*, 2002; Berg *et al.*, 2010). Therefore, theory predicts that herbivore consumption rates increases exponentially with increased temperature (O'Connor *et al.*, 2011). However, once a species encounters temperatures beyond its thermal optimum, the consumption rates declines (Lemoine & Burkepile, 2012). Experimental studies have reported that rising temperatures may have highly variable effects on insect herbivory; for

example: increased herbivory in warmed plots in the field (Roy *et al.*, 2004; Liu *et al.*, 2011; de Sassi & Tylianakis, 2012) and in the lab (Kukal & Dawson, 1989; O'Connor, 2009), neutral effect of warming on herbivory (Richardson *et al.*, 2002) and even decreased herbivory with warming (Burt *et al.*, 2014). Over short timescales, warming may therefore destabilize community dynamics by increasing or decreasing feeding rates.

It is well-known that neighbouring plants affect the herbivore damage to a focal plant (Root, 1973; Barbosa *et al.*, 2009; Underwood *et al.*, 2014). Neighbours can either increase (associational susceptibility) or decrease (associational resistance) herbivore attraction (Tahvanainen & Root, 1972). Also the relative frequency of plant species in the neighbourhood and plant density can affect the plant-herbivore interaction. The density of conspecific neighbours, for example, can both increase or decrease herbivore load and feeding behaviour; these are referred to as resource concentration effects (Root, 1973) or dilution effects (Otway *et al.*, 2005), respectively. Hence, warming can indirectly influence plant-herbivore interactions via effects on neighbouring plants and these indirect effects of warming may enhance or counteract the direct effects.

While the impact of climate change at the single species level is clear, the impact at the community level requires further investigation because results from single-species experiments have to be scaled up to understand the effects of climate change on community composition and ecosystem functioning. Therefore, community-scale experiments are needed, preferably with multiple trophic levels. This study investigated the effect of warming on a simple community consisting of three species: rosy apple aphid *Dysaphis plantaginea* Passerini (Hemiptera: Aphididae) feeding on plantain, *Plantago lanceolata* L., and a heterospecific neighbouring plant species, perennial ryegrass, *Lolium perenne* L. The aphid does not feed on *L. perenne*. The experimental design consisted of monocultures and mixtures of *L. perenne* and *P. lanceolata* at three temperatures levels. *P. lanceolata* plants were subjected to herbivory by the aphid *D. plantaginea*. Our goals were to investigate the effects of warming on each of the species and on the interactions between them.

4.3 MATERIAL AND METHODS

4.3.1 Study species

The rosy apple aphid *D. plantaginea* is an important apple pest in Europe and North America. *D. plantaginea* overwinters as eggs on apple trees, the primary host plant, and migrates in spring to the obligate alternate hosts, *Plantago major* L. and *P. lanceolata* (Alford, 2014). On *Plantago* spp., they give birth to apterous (wingless) morphs that reproduce by parthenogenesis (Blommers *et al.*, 2004). Laboratory cultures of *D. plantaginea* were established for several years from individuals originating from a wild population in Avignon, France. The aphids were reared in small cages on *P. lanceolata* under laboratory conditions of 22 ± 1 °C.

We used two common grassland species, *L. perenne*, a perennial hemicryptophyte that grows in dense tussocks (Beddows, 1967), and *P. lanceolata*, a rosette-forming perennial forb (Sagar & Harper, 1964). Both species originate from a wild population in England. *L. perenne* is not a host plant for *D. plantaginea*.

4.3.2 Experimental setup

P. lanceolata and *L. perenne* were grown from seed on greenhouse benches under controlled laboratory conditions (16 h daylight : 8 h darkness and 22 ± 1 °C) and isolated from aphid infestations. The species were sown with a time lag of one week to prevent differences in size at the start of the experiment (Cotrufo & Gorissen, 1997) due to differences in germination rate. Two or three week-old seedlings were transplanted into 1.5 L pots, filled with sandy soil (93.2% sand, 4.6% silt, 2.2% clay; field capacity 0.13 $m^3\ m^{-3}$; pH 7.6; Kjeldahl-N 0.42 g kg^{-1}; 1% C in humus). The pots were randomly placed in environmentally controlled growth chambers, with three chambers for each of the three temperature treatments: 17 °C, 20 °C and 23 °C. Temperatures were chosen to reflect the range of potential increase in the next century, with the lowest temperature corresponding to the average temperature of a summer day in Belgium. Each temperature treatment consisted of 25 plant communities (pots) with three different plant compositions: (1) 5 monocultures of *L. perenne*; (2) 10 monocultures of *P. lanceolata*; and (3) 10 mixtures of both plant species in a 50:50 ratio. Each

community contained four individuals because we chose a replacement design to study the effect of interspecific competition on plant-herbivore interaction under warming. The plants were watered every two days according to the 10-year average of 14-15 raining days per month during the growing season. The quantities of water supplied to the pots (65 ± 5 ml) were calculated from the amount of rainfall during the summer months in Ghent. All pots received the same amounts of water so that any enhanced consumption of water would result in soil drought. All communities were fertilized with 10 g m^{-2} NH_4NO_3, 5 g m^{-2} P_2O_5, 10 g m^{-2} K_2O and micro-elements (Fe, Mn, Zn, Cu, B, Mo). The fertilizer was given dissolved in water in four equal amounts.

Plants received artificial light, with 16 h daylight : 8 h darkness photoperiod regime. In order to compensate for potential light differences within and between chambers, plants were rotated weekly between all chambers and plant positions within chambers were simultaneously randomized. During infestation, all pots were individually enclosed with a 40 cm-tall transparent plastic cylinder covered with a lightweight netting to ensure aphids did not migrate between pots. This infrastructure did not appear to physically limit plant growth. However, shading and low light levels underneath the cages were unavoidable.

We controlled for temperature effects on the initial biomass production of both plant species by exposing the monocultures and mixtures of the three temperature treatments to the same number of growing degree days before the start of the infestation. We preferred to simulate synchrony between the phenology of the herbivore and its host rather than an ecologically mismatched interaction, i.e. an induced asymmetry between plant and aphid biomass at the onset of the experiment. The aphids were introduced on *P. lanceolata* plants 1508 growing degree days from the start of the experiment in each temperature treatment. Growing degree days were calculated from the temperature of the chambers using the Baskerville and Emin (1969) method, applying a base growth temperature (the threshold temperature below which the rate of development is considered to be insignificant) of 4 °C (Grant, 1968). In each temperature treatment, five monocultures of *P. lanceolata* and five mixtures were randomly chosen for aphid infestation. At the start of the infestation, three adult, apterous aphids were placed with a dry paintbrush on the apex of each *P. lanceolata* plant in monocultures and

mixtures. Consequently, at the start of the infestation each pot contained 12 (monocultures) or 6 (mixtures) aphids. Pots that did not receive aphids acted as control pots.

4.3.3 Data collection

Aphid populations were counted daily. The aphids were collected when the population on the monocultures had reached 300 aphids on average. Consequently, the harvest time of the aphids depended on the temperature treatment (earlier at 23 °C than at 17 °C). All remaining aphids were transferred to 70% ethanol and counted under a stereomicroscope to determine the final numbers. After counting, the aphid population from each pot was dried at 70 °C for 48 h and weighed. The critical number of aphids in monocultures matched the threshold value for dispersal of aphids when plant conditions are sub-optimal (Dixon, 1998). We thus terminated the experiment before the aphid populations would crash, to avoid compromising the measurement of plant responses. For statistical analysis, the total number of aphids per pot was divided by the number of *P. lanceolata* individuals in that pot.

We wanted to examine whether the recovery from an aphid infestation differed as a function of temperature using chlorophyll a fluorescence measurements (see below). Therefore, all plants were harvested after a recovery period of 10 days. During the harvest, aboveground parts were separated from belowground parts and live from dead biomass by species. Root and shoot were weighted fresh. We could not separate the roots of *L. perenne* and *P. lanceolata*, therefore only the belowground biomass of the monocultures was measured. All plant material was dried at 70 °C for 48 h, and weighed again. The relative water content of the shoots was calculated as the difference between fresh and dry weight divided by the fresh weight. For statistical analysis, the sum of aboveground biomass per species was divided by the number of plants of that species in each pot. Total leaf area of *P. lanceolata* in control pots was determined with a portable area meter (LI-3000A, LI-COR, NE, USA). *P. lanceolata* plants in control pots were ground in a mill, and three subsamples of each pot were analyzed for nitrogen content using a Flash 2000 Organic element analyser (Thermo Scientific, Bremen, Germany).

Chlorophyll a fluorescence, which can detect photosynthetic stress effects prior to visible leaf damage (Lichtenthaler & Miehe, 1997), was measured on the youngest fully expanded leaf of each plant species per pot. These measurements were performed prior to and after the infestation and at the end of the experiment. Readings were taken at the start of the daylight regime on 30-min dark-acclimated leaves with a Hansatech Plant Efficiency Analyzer (King's Lynn, Norfolk, UK), on the same day for all treatments. Maximum quantum yield of photosystem II was calculated as $F_v/F_m = (F_m-F_0)/F_m$ where F_v = variable fluorescence, F_m = maximum fluorescence and F_0 = steady state fluorescence.

4.3.4 Data analysis

To investigate the effect of a neighbouring plant species and warming on the plant-herbivore interaction, we fitted a structural equation model (SEM) (Grace, 2006; Lamb *et al.*, 2011) using the lavaan library in R (Rosseel, 2012; R Core Team, 2014). The response of individual aphids was measured as the generation time and the response of the population as the number of aphids at the population peak (see below). We hypothesized that (Fig. 1A):

- warming shortens the individual generation time of aphids. Shortening of generation time with increasing temperature is expected to enhance the growth rate of the population.
- warming decreases the leaf nitrogen and water content (An *et al.*, 2005; Flynn *et al.*, 2006; Jamieson *et al.*, 2012) and thus indirectly reduces the host plant quality for insect herbivores.
- interspecific competition in mixtures reduces the biomass of *P. lanceolata*. Therefore, in mixtures, *P. lanceolata* would experience more stress and be more vulnerable for aphids attack.

Because we control for the initial biomass at the start of the infestation (see experimental setup), we expect that warming would only slightly increase the biomass of *L. perenne* and *P. lanceolata* at the end of the experiment. Prior to fitting the SEM, we checked that relationships were linear using general linear models. We standardized live aboveground biomass of *L. perenne*, live aboveground biomass of *P. lanceolata* (control), leaf nitrogen, generation time, maximum number of aphids and live aboveground biomass of *P. lanceolata* (with aphids) by dividing raw values by the standard

deviation in order to equalize variances. We used the χ^2 goodness of fit statistic to test whether the covariance matrix generated by the model differed significantly from the data (a P-value > 0.05 indicates that the observed and expected covariance matrices are not significantly different, suggesting adequate model fit).

All data, except for leaf nitrogen, were also analyzed with ANOVA. Analyses were performed in SAS (version 9.4, SAS Institute Inc., Cary, NC) using General Linear Models (GLM). Several aphid population parameters were tested as a function of temperature and plant composition and plant responses as a function of temperature, plant composition and aphid infestation. Non-significant factors were always backwards excluded from the model. In case of significant effects, *a posteriori* means comparisons using Tukey test corrected for multiple comparisons were made. Effects were considered significant at $P \leq 0.05$.

The number of days between introduction of the adult aphid and the appearance of the first offspring was used as an approximation of generation time. However, in this study, the generation time corresponded with the settling time, the time that it takes the aphid to start feeding from the phloem. The maximum number of aphids (Nmax) equates the herbivory rate at a certain time point. For each population, an exponential growth curve was fitted through the aphid abundances from day one until the day of population peak. The growth constant k of the curve $N = N_0.e^{kt}$ served as a measure of population growth speed. Average aphid weight was determined by dividing population weight by population number.

The plant responses were tested separately for *L. perenne* and *P. lanceolata*. Relative herbivory effects were calculated as (live aboveground biomass of *P. lanceolata* with herbivores – live aboveground biomass of *P. lanceolata* without herbivores)/(live aboveground biomass of *P. lanceolata* without herbivores) and the relative biomass of *P. lanceolata* as (live aboveground biomass of *P. lanceolata* – live aboveground biomass of *L. perenne*)/(live aboveground biomass of *L. perenne)*. Live aboveground biomass and relative water content of the shoots were log-10 transformed, dead aboveground biomass was square root transformed and F_v/F_m was arcsine transformed to meet the data distribution assumptions. Leaf nitrogen was analysed with General Linear Mixed Models in SAS with temperature and

plant composition as fixed factors and pot as a random factor because we had three subsamples of each pot.

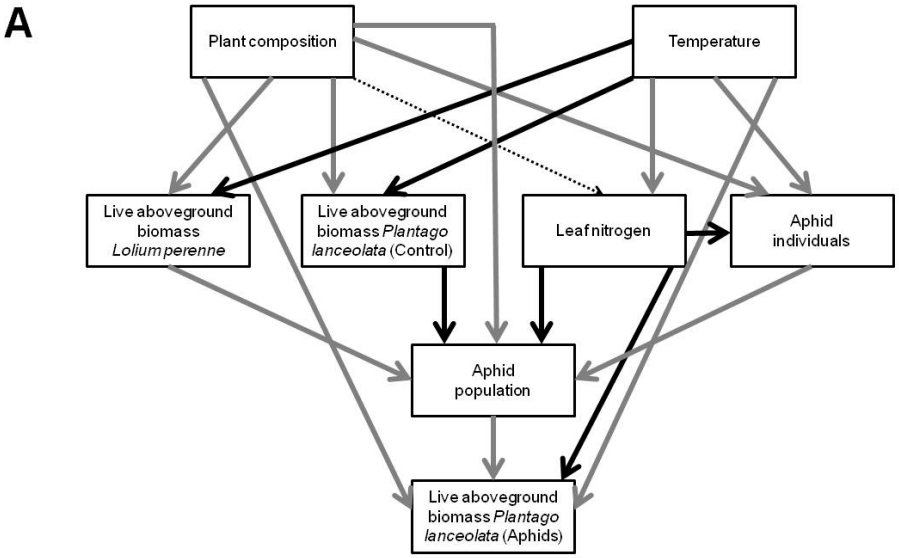
4.3.5 Data deposition

Data available from the Dryad Digital Repository: < http://dx.doi.org/10.5061/dryad.d5h06 > (Van De Velde *et al.*, 2016a).

4.4 RESULTS

4.4.1 Overview by SEM

The hypothesized structural relationship adequately fits the data (χ^2 = 17.044, df = 11 and P = 0.073). Fig. 1B and Table S1 (see supplementary material section 2) show that the following pathways were supported: 1) indirect paths from temperature via aphid individuals to aphid population, 2) direct paths from plant composition to live aboveground biomass of *L. perenne*, 3) direct path from plant composition to live aboveground biomass of *P. lanceolata* in control pots, and 4) direct path from temperature to leaf nitrogen. However, live aboveground biomass of *P. lanceolata* (in control pots) and leaf nitrogen did not affect aphid populations. Finally, neither temperature, plant composition or leaf nitrogen, nor aphid population had an influence on live aboveground biomass of *P. lanceolata* (with aphids). Summarizing these results, we conclude that temperature directly affected aphids by shortening the generation time. Shorter generation time in turn, increased the aphid population.


A
Plant composition
Temperature
Live aboveground biomass *Lolium perenne*
Live aboveground biomass *Plantago lanceolata* (Control)
Leaf nitrogen
Aphid individuals
Aphid population
Live aboveground biomass *Plantago lanceolata* (Aphids)

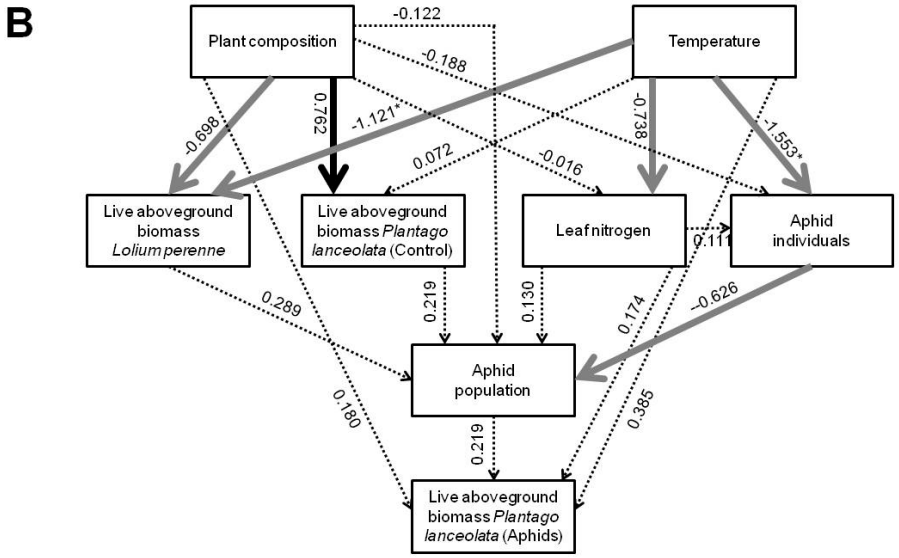

B
Plant composition
Temperature
-0.122
-0.188
-0.698
0.762
-1.121*
0.072
-0.016
-0.738
-1.553*
Live aboveground biomass *Lolium perenne*
Live aboveground biomass *Plantago lanceolata* (Control)
Leaf nitrogen
Aphid individuals
0.111
0.289
0.219
0.130
0.174
-0.626
Aphid population
0.180
0.219
0.385
Live aboveground biomass *Plantago lanceolata* (Aphids)

Fig. 1 (A) Specific predictions and (B) structural equation model showing how temperature and plant composition affect aphid population and the final live aboveground biomass of *Plantago lanceolata*. Solid arrows represent significant relationships ($P < 0.05$), dashed lines are nonsignificant. Black arrows are positive relationships, grey lines negative. Standardized path coefficients are shown next to pathways. For the effect of temperature, the average path coefficients are shown. The individual path coefficients of high and moderate warming can be seen in Table S1 (see supplementary material section 2). Significant effects of both high and moderate warming are indicated with an asterisk ($P < 0.05$). Live aboveground biomass of *Lolium perenne*, live aboveground biomass of *P. lanceolata* (control), leaf nitrogen, generation time, maximum number of aphids and live aboveground biomass of *P. lanceolata* (with aphids) were scaled before analysis.

4.4.2 Detailed analyses of the separate paths by linear models

Temperature, but not plant composition or leaf nitrogen, altered the generation time of aphids (Fig. 1B; Table 1). It was shorter at 20 °C compared to 17 °C but increased again at 23 °C (Fig. 2A). As expected, a shorter generation time increased the population, measured as Nmax (Fig 1B; Table S1, see supplementary material section 2). Furthermore, Nmax differed significantly according to an interaction between temperature and plant composition (Fig. 2B; Table 1). Temperature did not alter Nmax of monocultures because we artificially defined it. However, Nmax of monocultures act as controls for mixtures. Pairwise comparisons revealed that competition between *L. perenne* and *P. lanceolata* at 17 °C significantly decreased Nmax but increased it at 20 °C and did not alter it at 23 °C. In line with Nmax, also the aphids' population growth constant differed significantly according to an interaction between temperature and plant composition (Fig. 2C; Table 1). The aphids' population growth in monocultures was significantly higher at 23 °C compared to 17 °C and 20 °C. However, in mixtures, the growth increased significantly at 20 °C and remained higher at 23 °C. Again, pairwise comparisons revealed that competition between *L. perenne* and *P. lanceolata* decreased the aphids' population growth at 17 °C but increased it at 20 °C and did not alter the growth at 23 °C. In addition, the average aphid weight peaked at 20 °C but remained unaffected by plant composition (Fig. 3; Table 1). We conclude that aphid populations on *P. lanceolata* at 20 °C were characterised by stronger exponential growth, short generation times, larger aphids and larger maximum population size than at 17 °C. This pattern was most obvious in mixtures.

Table 1 Summary of ANOVA results for effects of temperature and plant composition on aphid performance growing on *Plantago lanceolata*. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*. P values are presented in bold when significant (<0.05).

Measurement	Treatment	df	F	P
Generation time	Temperature	2,24	27.17	**<0.001**
	Plant composition	1,24	0.77	0.389
	Temperature × Plant composition	2,24	2.43	0.109
Maximum number of aphids	Temperature	2,24	3.66	**0.041**
	Plant composition	1,24	0.02	0.892
	Temperature × Plant composition	2,24	6.57	**0.005**
Exponential growth constant	Temperature	2,24	28.5	**<0.001**
	Plant composition	1,24	0.07	0.796
	Temperature × Plant composition	2,24	7.19	**0.004**
Average aphid weight	Temperature	2,24	8.27	**0.002**
	Plant composition	1,24	0.49	0.490
	Temperature × Plant composition	2,24	1.22	0.280

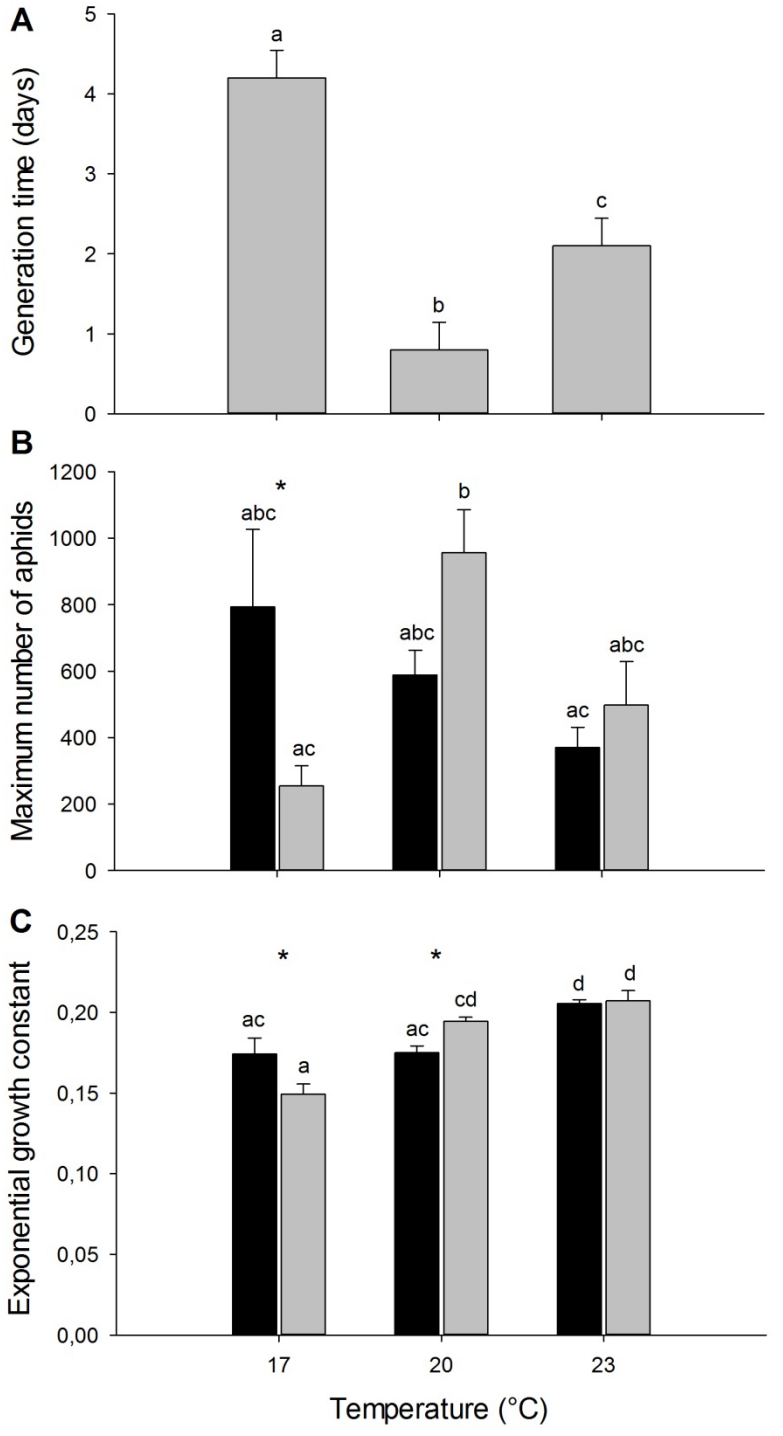

A
Generation time (days)
5
4
3
2
1
0
a
b
c
B
Maximum number of aphids
1200
1000
800
600
400
200
0
*
abc
ac
abc
b
ac
abc
C
Exponential growth constant
0,25
0,20
0,15
0,10
0,05
0,00
*
*
ac
a
ac
cd
d
d
17
20
23
Temperature (°C)

Fig. 2 Effect of temperature and plant composition on aphid population dynamics. A) Effect of temperature on the generation time of aphids (all plant compositions combined). B) Effect of temperature and plant composition on the maximum number of aphids. C) Effect of temperature and plant composition on the growth constant k of the exponential growth curve. Bars represent means ± SE. Plant communities consist of monocultures of *Plantago lanceolata* (black bars) and mixtures of *Lolium perenne* and *P. lanceolata* (grey bars). Significant pairwise differences are indicated by different letters above the bars (P <0.05). Significant differences between monocultures and mixtures at a given temperature are indicated with an asterisk (P <0.05).

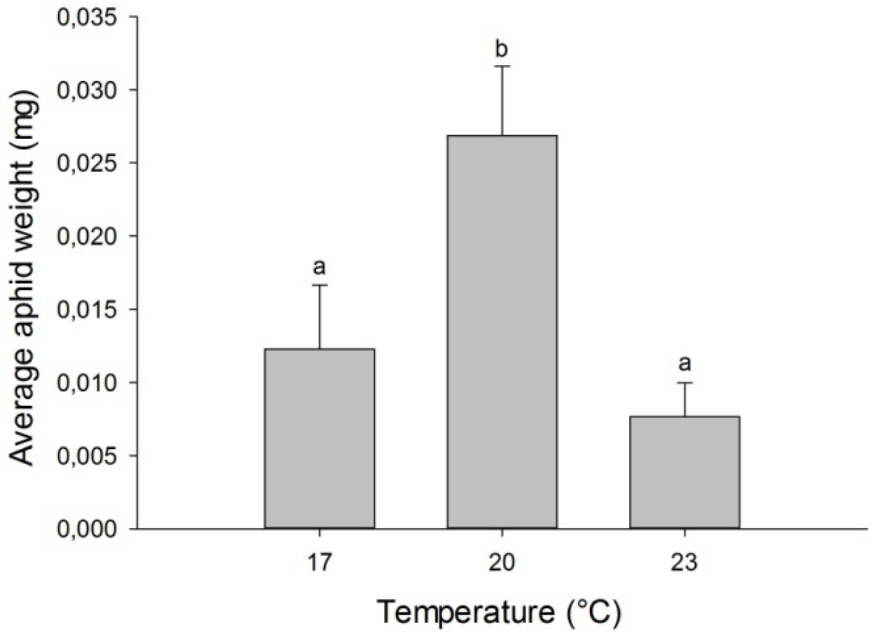


Fig. 3 Effect of temperature on average aphid weight (mean ± SE, all plant compositions combined). Significant pairwise differences are indicated by different letters above the bars (P <0.05).

Aphid infestation reduced considerably the relative aboveground biomass ($F_{1,24}$ = 14.35, P = 0.0009), the live aboveground biomass, the belowground biomass and the relative water content of the shoots of *P. lanceolata* (Fig. 4A, Fig. 5C, Fig. S1, see supplementary material section 1; Table 2). Concurrently, *P. lanceolata* infested with aphids showed reduced F_v/F_m at the end of the infestation ($F_{1,48}$ = 8.58, P = 0.0051, Fig. S2, see supplementary material section 1) and F_v/F_m of infested plants dropped further at the end of the experiment ($F_{1,48}$ = 48.16, P <0.0001, Fig. S2, see supplementary material section 1). This indicated that the plants did not recover from the aphid infestation. In addition, aphid infestation increased the dead aboveground biomass of *P. lanceolata* (Fig. 4B; Table 2) and the live aboveground biomass of *L. perenne* in mixtures (Fig. 4C; Table 2).

Temperature and plant composition did not alter the live aboveground biomass and, relative water content of the shoots of *P. lanceolata* (Fig. 4A; Table 2), nor the relative herbivory effects ($F_{2,26}$ = 0.46, P = 0.6361; $F_{1,26}$ = 0.0, P = 0.9841 respectively, Fig. 5A) at the end of the experiment. However, at 23 °C there was more *P. lanceolata* biomass with respect to *L. perenne* in mixtures (aphid treatment and controls combined), whereas the opposite was true at 17 °C ($F_{2,24}$ = 6.13, P = 0.0071, Fig. 5B). Furthermore, in controls, the live aboveground biomass of *P. lanceolata* was significantly higher in mixtures compared to monocultures irrespective of the temperature (Fig. 1B, Fig. 4A). The dead aboveground biomass of *P. lanceolata*, on the other hand, differed significantly according to an interaction between temperature and plant composition (Fig. 4B; Table 2). This was mainly due to a significant increase in dead biomass in monocultures compared to mixtures at 17 °C. The belowground biomass increased at 20 °C, but decreased again at 23 °C to similar levels as 17 °C (Fig. 4B, Fig. S1, see supplementary material section 1; Table 2). In contrast, leaf nitrogen of *P. lanceolata* decreased slightly at 20 °C (Fig. 6; Table 2). The specific leaf area was significantly higher at 23 °C compared to the other temperature treatments (Fig. S3, see supplementary material section 1; Table 2). Before infestation, F_v/F_m of *P. lanceolata* was slightly lower at 17 °C compared to the other temperature treatments ($F_{2,48}$ = 7.40, P = 0.0014), but temperature did not alter F_v/F_m after infestation and at the end of the experiment. We conclude that aphid infestation and temperature had more effect on *P. lanceolata* compared to plant composition.

Temperature affected all measured plant responses of *L. perenne* (Table 2). Notably, its live aboveground biomass, the shoot relative water content and surprisingly the dead aboveground biomass decreased with increasing temperature (Fig. 4C, Fig. 4D; Table 2). In line with *P. lanceolata* responses, the belowground biomass of *L. perenne* peaked at 20 °C and decreased again at 23 °C to reach similar levels as at 17 °C (Fig. S4, see supplementary material section 1; Table 2). Before infestation, F_v/F_m was higher at 20 and 23 °C compared to 17 °C ($F_{2,36}$ = 16.13, P <0.0001, Fig. S5, see supplementary material section 1). However, after infestation ($F_{2,36}$ = 7.82, P = 0.0015, Fig. S5, see supplementary material section 1) and at the end of the experiment ($F_{2,36}$ = 4.29, P = 0.0201, Fig. S5, see supplementary material section 1), F_v/F_m dropped slightly at 20 °C compared to 17 °C and 23 °C. Competition with *P. lanceolata* reduced the live aboveground biomass of *L. perenne* (irrespective of the temperature) and the dead aboveground biomass but only at 20 °C (Fig. 4C; Table 2).

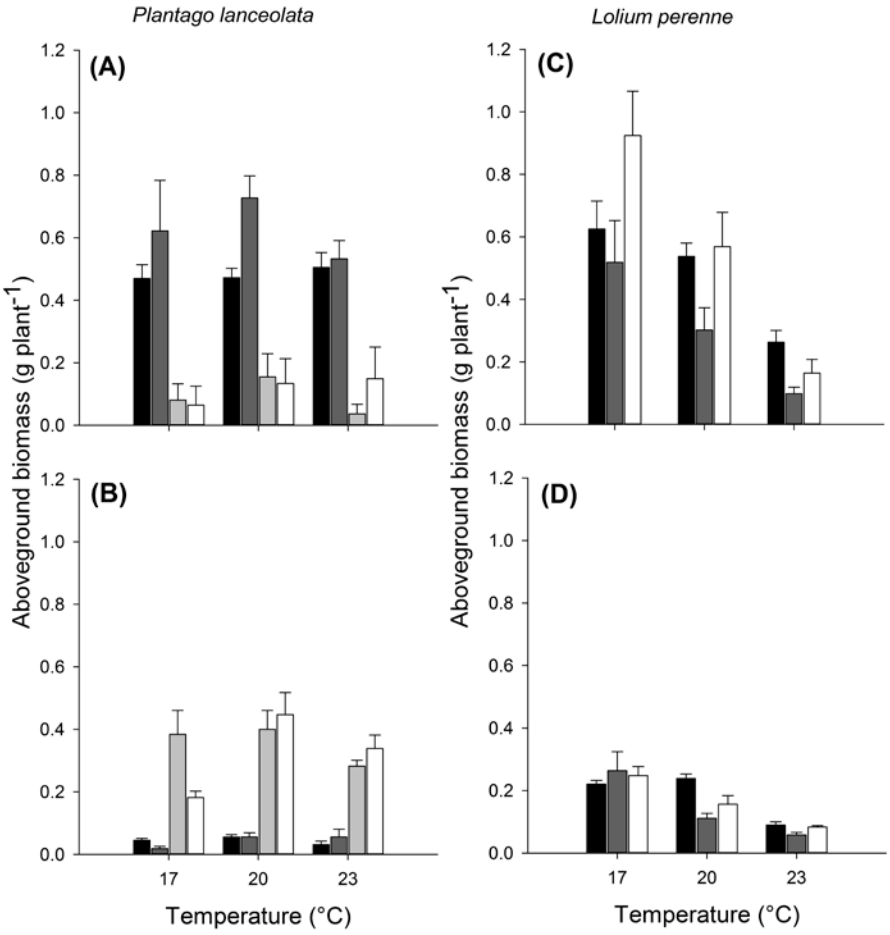


Fig. 4 Effect of temperature, plant composition and aphid infestation on A) the live aboveground biomass of *Plantago lanceolata*, B) the dead aboveground biomass of *P. lanceolata*, C) the live aboveground biomass of *Lolium perenne* and D) dead aboveground biomass of *L. perenne*. Bars represent means ± SE. Plants were grown in monocultures of *L. perenne* or *P. lanceolata* (black bars), mixtures of *L. perenne* and *P. lanceolata* (dark grey bars), monocultures with aphids (light grey bars) or mixtures with aphids (white bars).

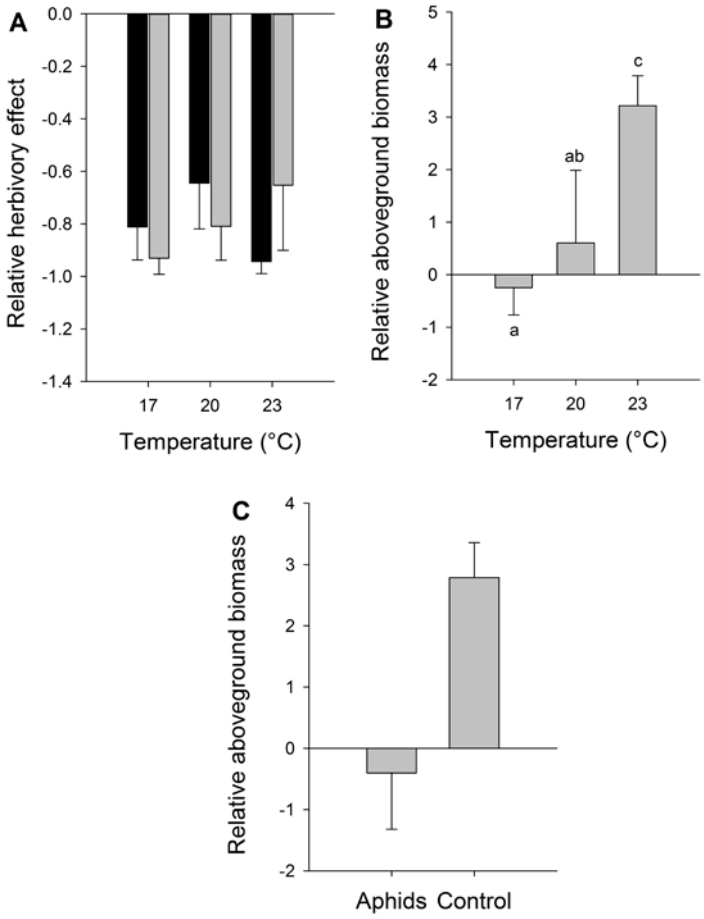


Fig. 5 Relative change in plant biomass of *Plantago lanceolata* due to warming and aphid herbivory. A) Aboveground biomass effects of herbivory on *P. lanceolata* exposed to different temperatures relative to controls. The relative herbivory effect was calculated as (live aboveground biomass of *P. lanceolata* with herbivores – live aboveground biomass of *P. lanceolata* without herbivores)/(live aboveground biomass of *P. lanceolata* without herbivores). Plant communities consisted of monocultures of *P. lanceolata* (black bars) and mixtures of *Lolium perenne* and *P. lanceolata* (grey bars). B) Effect of temperature and C) effect of aphid infestation on aboveground biomass of *P. lanceolata* relative to aboveground biomass of *L. perenne*. The relative aboveground biomass of *P. lanceolata* was calculated as (live aboveground biomass of *P. lanceolata* – live aboveground biomass of *L. perenne*)/(live aboveground biomass of *L. perenne*). Bars represent means ± SE. Significant pairwise differences are indicated by different letters above the bars ($P < 0.05$).

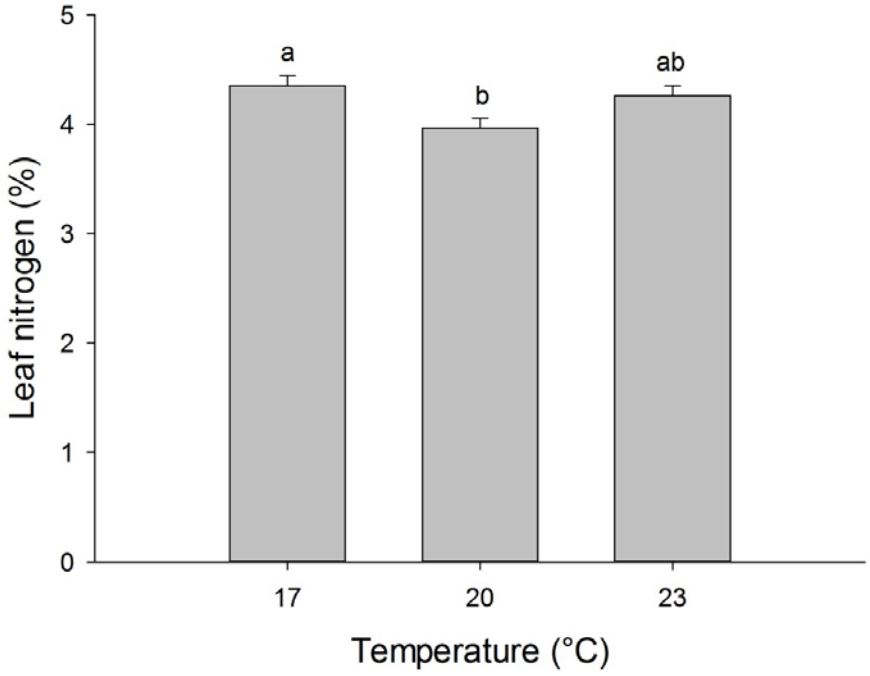


Fig. 6 Effect of temperature on percentage of nitrogen in leaves of *Plantago lanceolata* that did not receive aphids. Bars represent means ± SE are indicated (all plant compositions combined). Significant pairwise differences are indicated by different letters above the bars ($P < 0.05$).

Table 2 Summary of ANOVA results for effects of temperature, plant composition and aphid infestation on plant performance. Plant communities consist of monocultures and mixtures of *Lolium perenne* and *Plantago lanceolata*. P values are presented in bold when significant (<0.05).

		Plantago lanceolata			*Lolium perenne*		
Measurement	Treatment	df	F	P	df	F	P
Live aboveground biomass	Temperature	2,48	1.09	0.345	2,36	22.23	**<0.001**
	Plant composition	1,48	2.66	0.120	1,36	14.18	**0.006**
	Infestation	1,48	118.4	**<0.001**	1,36	10.97	**0.002**
	Temperature × infestation	2,48	0.05	0.952	2,36	0.2	0.822
	Temperature × plant composition	2,48	0.12	0.892	2,36	1.68	0.201
	Plant composition × infestation	1,48	1.11	0.298	-	-	-
	Temperature × infestation × plant composition	2,48	1.37	0.264	-	-	-
Dead aboveground biomass	Temperature	2,48	7.08	**0.002**	2,36	23.45	**<0.001**
	Plant composition	1,48	1.13	0.292	1,36	6.88	**0.013**
	Infestation	1,48	302.78	**<0.001**	1,36	2.18	0.148
	Temperature × infestation	2,48	0.77	0.468	2,36	0.74	0.484

		Plantago lanceolata			*Lolium perenne*		
Measurement	Treatment	df	F	P	df	F	P
	Temperature × plant composition	2,48	7.01	**0.002**	2,36	5.29	**0.010**
	Plant composition × infestation	1,48	0.24	0.630	-	-	-
	Temperature × infestation × plant composition	2,48	0.81	0.450	-	-	-
Belowground biomass	Temperature	2,24	39.34	<**0.001**	2,12	47.35	<**0.001**
	Infestation	1,24	43.67	<**0.001**	-	-	-
	Temperature × infestation	2,24	1.82	0.183	-	-	-
Relative water content of the shoots	Temperature	2,44	0.36	0.702	2,36	4.4	**0.020**
	Plant composition	1,44	1.61	0.211	1,36	0.69	0.410
	Infestation	1,44	27.79	<**0.001**	1,36	0.35	0.558
	Temperature × infestation	2,44	0.45	0.641	2,36	0.18	0.836
	Temperature × plant composition	2,44	1.81	0.175	2,36	0.25	0.780
	Plant composition × infestation	1,44	2.82	0.100	-	-	-
	Temperature × infestation × plant composition	2,44	1.81	0.176	-	-	-

		Plantago lanceolata			*Lolium perenne*		
Measurement	Treatment	df	F	P	df	F	P
Leaf nitrogen	Temperature	2,24	4.93	**0.016**	-	-	-
	Plant composition	1,26	0.04	0.834	-	-	-
	Temperature × plant composition	2,27	0.71	0.501	-	-	-
Specific leaf area	Temperature	2,24	12.2	**0.001**	-	-	-
	Plant composition	1,24	1.26	0.272	-	-	-
	Temperature × plant composition	2,24	0.98	0.391	-	-	-

4.5 DISCUSSION

To understand the impact of climate warming on the complex networks of species in communities, species interactions need to be considered. We investigated the effect of warming on a model community consisting of an aphid feeding on *P. lanceolata* and a heterospecific neighbouring plant species *L. perenne*. Warming affected the aphid's performance directly, but not indirectly through changes in host plant quality. Aphids performed best at moderate warming.

4.5.1 Direct effect of warming on aphid performance

As expected, experimental warming directly affected the life history traits of the aphid *D. plantaginea*, though in a non-linear manner. Aphid populations at 20 °C in mixtures were characterised by shorter generation times, stronger exponential growth, larger aphids and larger maximum population sizes compared to 17 °C. Therefore, 20 °C may be the upper thermal threshold for the aphid *D. plantaginea*. Generally, above the upper temperature threshold, activity costs are higher, inducing behavioural and physiological changes. Indeed, at 23 °C the observed generation time was longer and Nmax and aphid weight were lower. Yet, this level of warming still accelerated the exponential growth of the population by means of higher fecundity (Meisner *et al.*, 2014; Ramalho *et al.*, 2015). Probably, higher mortality caused by exposure to stressful temperatures underlies the observed lower Nmax despite of the faster exponential growth at 23 °C. This would be in line with the theory that mortality increases when temperature exceeds the optimal range (Amarasekare & Savage, 2012).

The relative biomass losses of *P. lanceolata* due to insect herbivores were not altered with warming. Therefore, the higher dry weight of aphids at 20 °C points towards a functional instead of numerical response of the aphids with moderate warming (Solomon, 1949; Holling, 1959; Holling, 1965). At that temperature, aphids grew faster probably due to a higher efficiency in converting food into body matter.

4.5.2 Indirect effect of warming on aphid performance

Insect herbivores are influenced by the food quality of the plant material they consume (Mattson, 1980; Awmack & Leather, 2002). In aphids, reproduction depends on the nutritional status and availability of the host plant (Dixon, 1998; Awmack & Leather, 2002). Therefore, warming might alter aphid performance also indirectly through bottom-up effects, by changing host plant availability and quality. In the current study, however, we have controlled for temperature effects on the initial biomass production in order to exclude a different carrying capacity. Warming did not alter the biomass production of *P. lanceolata* at the end of the experiment; hence the faster exponential growth of the aphids at higher temperature cannot originate from more available food. It cannot arise from an altered leaf nitrogen status either, since nitrogen content was slightly lower at 20 °C compared to 17 °C, increasing again to the control value at 23 °C. Warming has been shown to decrease leaf nitrogen content in earlier studies (An *et al.*, 2005; Flynn *et al.*, 2006), thus reducing host plant quality for insect herbivores, but our structural equation model showed that leaf nitrogen did not affect the aphids. Therefore, the increased exponential growth of aphids at higher temperatures must be due to direct temperature effect.

On the other hand, leaf nitrogen content could be a poor index of nutritional value since aphids depend more on the soluble amino acids in the phloem (Schoonhoven *et al.*, 2005). We can therefore not exclude that changes in the quality rather than the quantity of nitrogen-based compounds in the phloem, or changes in other plant nutrients than the one we measured such as phosphorus or potassium (Jansson & Ekbom, 2002), may have contributed to the observed faster growth rates at higher temperature. In addition, the water content of foliage can also have an effect on the growth of aphids (Schoonhoven *et al.*, 2005). Yet, we found no effect of warming on the relative water content of *P. lanceolata* shoots. All in all, we found fewer indications for indirect than direct effects of warming on aphid performance.

4.5.3 Effect of plant species composition

Interspecific competition decreased the live aboveground biomass of *L. perenne* and increased the live aboveground biomass of *P. lanceolata* in the controls. In mixtures, *L. perenne* may have absorbed fewer nutrients

compared to monocultures which may have resulted in more available nutrients for *P. lanceolata*. In addition, interspecific competition affected maximum population size and exponential growth rate of the aphids through an interaction with temperature. Interspecific competition at 17 °C negatively affected the performance of the aphids by reducing their population growth rate and maximum population size compared to 20 °C. By contrast, the opposite was observed at 20 °C but not at 23 °C. We expected a higher aphid performance under interspecific competition irrespective of temperature as the growth-differentiation balance hypothesis predicts reduced defence against herbivores under interspecific competition, owing to greater investment of energy in "defence" against competitors (Herms & Mattson, 1992). Pellissier *et al.* (2014) demonstrated that temperature affects secondary metabolite production in *P. lanceolata*, which are well-known for their role in plant defence against insect herbivory. In *P. lanceolata* the secondary plant compound iridoid glycosides increased in response to herbivory which negatively influenced both its specialist and generalist insect herbivores (Bowers *et al.*, 1992). Low temperatures can constrain the induction of iridoid glycosides and therefore reduce the resistance against herbivory. Today it is not clear how interspecific competition and temperature interact to affect plant defence. Further experimentation is necessary to untangle these factors and their ultimate influence on herbivores. In conclusion, we showed that plant composition and temperature interacted to affect aphid performance but the mechanism at the basis of the observed patterns requires elucidation.

4.5.4 Effect of warming on herbivory rates

The herbivory rates on *P. lanceolata* were quantified as relative changes in plant biomass due to insect herbivores. In this study, the aphids performed best at moderate warming, where they grew faster and had a shorter generation time. Despite of this, the relative biomass losses of *P. lanceolata* did not alter under warming and consequently the net interaction strength between plants and herbivores was not changed under warming. This finding points to reduced consumption rates at higher temperature which may result from metabolic demand exceeding energetic supply, such that energy available for tasks beyond cellular maintenance, such as digestion, feeding and, movement, decreases sharply at high temperatures (Somero, 2011).

However, our finding that warming did not affect the herbivory rates is in contrast to theoretical studies which predict that ectothermic herbivores must increase food intake at higher temperatures to offset increased metabolic or nutritional demand (O'Connor *et al.*, 2001). As a result, herbivory rates should increase exponentially with rising temperature, more than primary production, reducing plant biomass at higher temperatures (Gillooly *et al.*, 2001; O'Connor, 2009; O'Connor *et al.*, 2011). Lemoine *et al.* (2014) concluded that the effect of temperature on herbivory rates are highly variable, depending on the identity of the herbivore-plant combination.

4.5.5 Conclusion

We found warming and aphid herbivory to alter plant community composition but not net interaction strength between plants and herbivores within the simplified experimental community. This is in contrast to theoretical predictions (Gilbert *et al.*, 2014) that consider consumer-prey models and not plant-plant interactions. The stability of net interaction strength, suggests that the response of a simplified community to warming may scale up to understand the effect of warming on more complex community and ecological networks.

Our controlled laboratory experiment allowed us to precisely measure the effect of interspecific competition on plant-herbivore interaction under warming. Such single-factor climate experiments can improve mechanistic understanding because the low complexity makes isolating specific processes easier (De Boeck *et al.*, 2015). However, in the field, plant communities are subjected to multiple climate change drivers including also elevated CO_2 and altered water conditions. These factors can also interact and multifactor climate experiments have shown that combined responses can be smaller than those expected from additive, single-factor effects (Wu *et al.*, 2011). Moreover, in natural grassland usually more species are present. Therefore, future studies need to validate whether net interactions strengths also remain stable with multiple climate change drivers in more complex communities in natural ecosystems. To conclude, this proof-of-concept study provides evidence that the net interactions with herbivores should not change under warming despite direct effects of warming on herbivores. Therefore, our study stresses the importance of indirect non –

trophic interactions as an additional layer of complexity to improve our understanding of how trophic interactions will alter under climate change.

4.6 ACKNOWLEDGEMENTS

H. Van De Velde is a Research Assistant of the Fund for Scientific Research-Flanders (FWO). D. Bonte was funded by the FWO research network "An eco-evolutionary network of biotic interactions". We thank the Earth and Life Institute (Université Catholique de Louvain) for providing *Dysaphis plantaginea*.

4.7 SUPPLEMENTARY MATERIAL

4.7.1 Section 1: supplementary figures

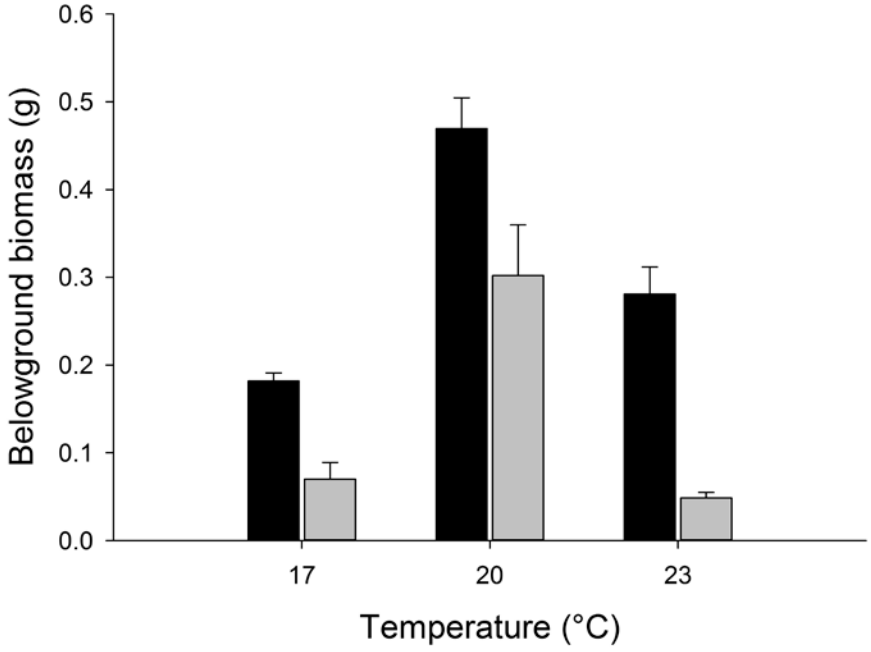


Fig. S1 Effect of temperature and aphid infestation on belowground biomass of *Plantago lanceolata*. Mean ± SE are indicated (all plant compositions combined). Black bars: controls and grey bars: aphid infestation.

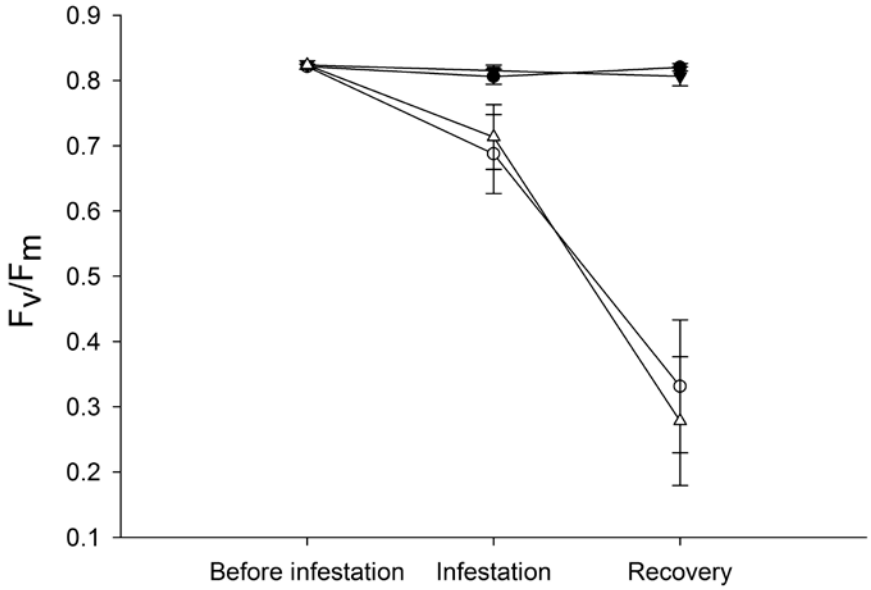


Fig. S2 Effect of plant composition and aphid infestation on F_v/F_m of *Plantago lanceolata* before aphid infestation, just after aphid infestation and after recovery. Mean ± SE are indicated. Plant communities consisted of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*. F_v/F_m represents the maximum quantum yield of photosystem II. F_v = variable fluorescence, F_m = maximum fluorescence. Black circle = monocultures; white circle = monocultures + aphid infestation; black triangle = mixtures and white triangle = mixtures + aphid infestation.

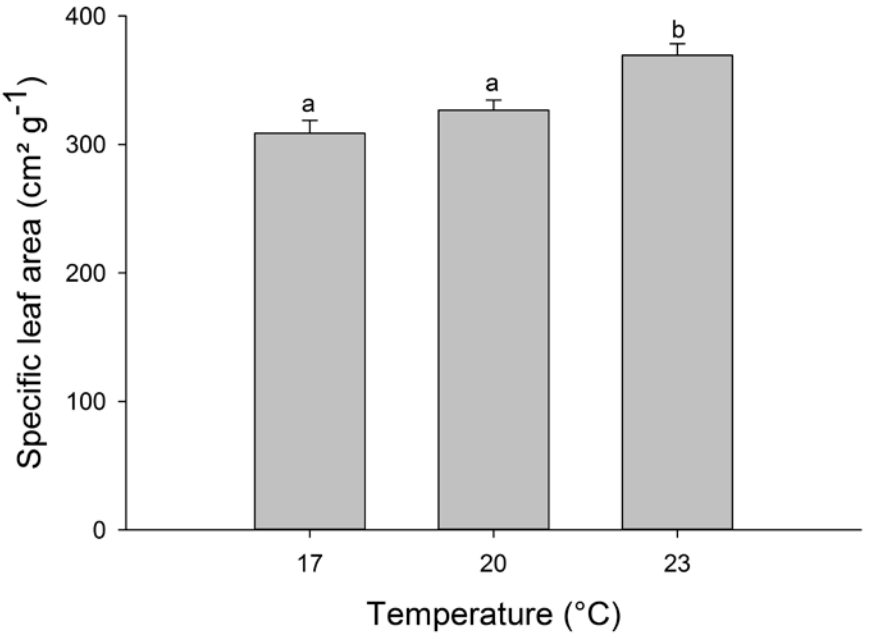


Fig. S3 Effect of temperature on specific leaf area of *Plantago lanceolata* plants that did not receive aphids. Mean ± SE are indicated (all plant compositions combined). Significant pairwise differences are indicated by different letters above the bars ($P < 0.05$).

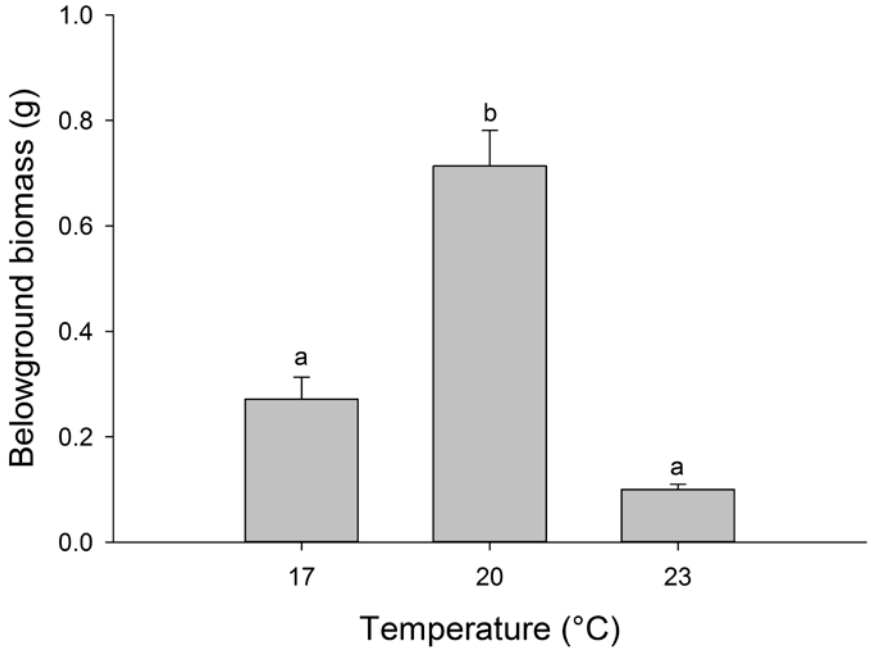


Fig. S4 Effect of temperature on belowground biomass of *Lolium perenne*. Mean ± SE are indicated (all plant compositions combined). Significant pairwise differences are indicated by different letters above the bars ($P < 0.05$).

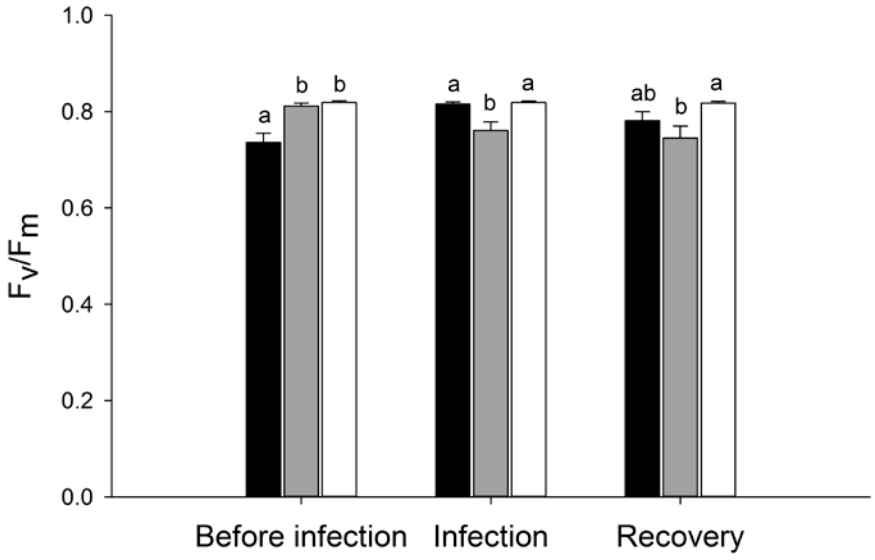


Fig. S5 Effect of temperature on F_v/F_m of *Lolium perenne* before aphid infestation, just after aphid infestation and after recovery. Mean ± SE are indicated (all plant compositions combined). Significant pairwise differences between temperature treatments are indicated by different letters above the bars ($P < 0.05$). Black bars = 17 °C, grey bars = 20 °C and white bars = 23 °C.

4.7.2 Section 2: supplementary table

Table S1 Partial slopes of the structural equation model presented in Figure 1B. Plant communities consist of monocultures and mixtures of *Lolium perenne* and *Plantago lanceolata*. Moderate warming represents the effect of increasing temperature from 17 °C to 20 °C and high warming from 17 °C to 23 °C. P values are presented in bold when significant (<0.05).

		Estimate	SE	Z-value	P-value
Live aboveground biomass *Plantago lanceolata* (Control)					
	Moderate warming	0.284	0.398	0.715	0.475
	High warming	-0.141	0.398	-0.353	0.724
	Plant composition	0.762	0.325	2.346	**0.019**
Leaf nitrogen	Moderate warming	-1.214	0.373	-3.259	**0.001**
	High warming	-0.261	0.373	-0.700	0.484
	Plant composition	-0.016	0.304	-0.053	0.957
Aphid population	Leaf nitrogen	0.130	0.168	0.775	0.438
	Live aboveground biomass *Plantago lanceolata* (Control)	0.219	0.161	1.362	0.173
	Live aboveground biomass *Lolium perenne*	0.289	0.167	1.731	0.083
	Aphid individuals	-0.626	0.177	-3.543	**0.000**
	Plant composition	-0.122	0.336	-0.364	0.716

		Estimate	SE	Z-value	P-value
Aphid individuals	Moderate warming	-1.785	0.296	-6.029	**0.000**
	High warming	-1.157	0.257	-4.51	**0.000**
	Plant composition	-0.186	0.208	-0.897	0.370
	Leaf nitrogen	0.111	0.125	0.891	0.373
Live aboveground biomass *Lolium perenne*	Moderate warming	-0.627	0.284	-2.206	**0.027**
	High warming	-1.614	0.284	-5.682	**0.000**
	Plant composition	-0.698	0.232	-3.009	**0.003**
Live aboveground biomass *Plantago lanceolata* (Aphids)	Leaf nitrogen	0.174	0.204	0.854	0.393
	Aphid population	0.219	0.187	1.175	0.240
	Plant composition	0.180	0.34	0.531	0.596
	Moderate warming	0.536	0.518	1.034	0.301
	High warming	0.234	0.421	0.556	0.579

5 INTERSPECIFIC PLANT COMPETITION MEDIATES IMPACT OF CLIMATE CHANGE ON PLANT-HERBIVORE INTERACTION

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Submitted to Global Change Biology

5.1 ABSTRACT

Analyzing the combined effect of warming and elevated CO_2 on biotic interactions is necessary for a better understanding of community responses to climate change. This study investigates such effects on a two-trophic model community consisting of three species: rosy apple aphid *Dysaphis plantaginea* feeding on plantain, *Plantago lanceolata*, and a heterospecific neighbouring plant species, perennial ryegrass, *Lolium perenne*. The aphid does not feed on *L. perenne*. We focused on the leaf quality and the chemical defence system and examined how both attributes influence insect herbivore performance. The experimental design consisted of monocultures of *P. lanceolata* and mixtures of *L. perenne* and *P. lanceolata* exposed to three simulated climate scenarios: (1) current climate, (2) elevated CO_2 and current temperature and (3) elevated CO_2 and warming. Elevated CO_2 reduced the leaf quality of *P. lanceolata*, while simultaneous increases of CO_2 and temperature modified the leaf quality in different directions depending on the stoichiometric component. Elevated CO_2 also enhanced both systemic and induced defence systems in *P. lanceolata*, and these effects were not changed by warming. However, when allowing for interspecific plant interactions from *L. perenne*, the positive effects of elevated CO_2 on the defence molecules in *P. lanceolata* were neutralised. We did not observe any direct effect of elevated CO_2, nor of its combination with warming, on aphids performance. Notwithstanding the significant effects of the future climate scenarios on leaf quality and defence molecules, we found no indications for indirect effects on aphid performance either. In this study, we demonstrate the importance of multispecies interactions as mediators of single species responses to climate change by either weakening or strengthening these single species responses.

5.2 INTRODUCTION

Climate change includes multiple factors such as increased atmospheric CO_2 and associated higher global mean temperatures. The independent effects of CO_2 and temperature on plant physiology and insect herbivores performance are well documented. Beyond the knowledge of climate change effects on focal species, it is pivotal to understand the impact of changing environmental conditions on interactions between species as every species is embedded in a complex web of interactions.

Interactions between insect herbivores and host plants are influenced by various primary and secondary metabolites; these metabolites may, in turn, be affected by climate change (Zvereva & Kozlov, 2006; Bidart-Bouzat & Imeh-Nathaniel, 2008). Plants exposed to a CO_2-enriched environment show higher concentrations of carbohydrates (including starch and soluble sugars), lower concentrations of nitrogen (either from dilution by increased carbohydrates or reallocation) and thus a higher C:N ratio (Lincoln *et al.*, 1993; Bezemer & Jones, 1998; Stiling & Cornelissen, 2007; Robinson *et al.*, 2012). Lower nitrogen concentration implies lower levels of leaf protein and amino acids and, as a result, reduced nutritive value to herbivores (Lincoln *et al.*, 1986). Consequently, in general, the performance of insects reduces (Lincoln *et al.*, 1993; Bezemer & Jones, 1998; Hunter, 2001). Contrary to elevated CO_2, warming has a direct positive effect on insect herbivore performance by reducing their development time (Bale *et al.*, 2002; Van De Velde *et al.*, 2016b) and increasing fecundity (Meisner *et al.*, 2014) up to a certain threshold. Yet, warming may decrease leaf nitrogen (An *et al.*, 2005; Flynn *et al.*, 2006; Zvereva & Kozlov, 2006) and may increase or decrease the level of carbohydrates, depending on whether photosynthesis is operating above or below its thermal optimum (DeLucia *et al.*, 2012). Therefore, warming may indirectly compromise the host plant quality for insect herbivores (Bauerfeind & Fischer, 2013; Jamieson *et al.*, 2015). Nevertheless, such indirect effects are unlikely to fully counterbalance the direct positive effects, and the overall herbivore response to warming will be positive (Zvereva & Kozlov, 2006; Bauerfeind & Fischer, 2013).

Secondary metabolites (e.g. lignin, tannins, phenolics and terpenoids) - although not required for primary plant metabolic processes such as nutrient assimilation or growth - affect the tissue quality by influencing the nutrition,

palatability, digestibility and/or toxicity of foliage. Compared with primary metabolites, the response of secondary metabolites to elevated CO_2 and warming is highly variable and less understood (Bidart-Bouzat & Imeh-Nathaniel, 2008; Robinson *et al.*, 2012). According to the carbon-nutrient balance hypothesis (Bryant *et al.*, 1983), elevated CO_2 are expected to increase carbon-based secondary compounds as a result of the 'excess' carbon and to decrease nitrogen-based compounds a result of the nitrogen scarcity (Robinson *et al.*, 2012). On the other hand, the growth-differentiation balance hypothesis (Herms & Mattson, 1992) proposes that warming-accelerated photosynthesis should contribute to growth rather than defence if resources (e.g. soil moisture and nutrients) are not limited. However, so far, studies suggest that the effects of warming and elevated CO_2 on the different groups of chemical defences are idiosyncratic (Zvereva & Kozlov, 2006; Bidart-Bouzat & Imeh-Nathaniel, 2008; Zavala *et al.*, 2013).

Additional complexity arises from competition with neighbouring plants. For example, competition for limited nutrients enhances the availability of carbon in a focal plant relative to its demand. This, in turn, can increase carbon-based defences and thus reduce herbivory compared with control plants that are less constrained by competitors (Bryant *et al.*, 1983). Also the identity of a neighbouring plant may affect the resistance of a focal plant by influencing the outcome of plant-plant competition (Barton & Bowers, 2006; Broz *et al.*, 2010). Conspecific competition, in particular, may result in stronger decline of plant growth and increased defence compared with heterospecific competition. Indeed, Broz *et al.* (2010) showed that a focal plant with conspecific neighbours allocated more resources towards production of carbon-based defence molecules, whereas those grown with heterospcific neighbours allocated more resources towards growth. Hence, elevated CO_2 as well as warming can indirectly influence defence on a focal plant, if their impact on neighbouring plants is different.

Although atmospheric CO_2 concentrations and temperature increase concurrently, empirical studies investigating their combined effect on multi-trophic communities are surprisingly scarce and insufficient to effectively guide theory or synthesis (Zvereva & Kozlov, 2006; Cornelissen, 2011). The current study investigates individual effects of elevated CO_2 and combined effects of elevated CO_2 and warming on a simple model community

consisting of three species: rosy apple aphid *Dysaphis plantaginea* Passerini (Hemiptera: Aphididae) feeding on plantain, *Plantago lanceolata* L., and a heterospecific neighbouring plant species, perennial ryegrass, *Lolium perenne* L. The aphid does not feed on *L. perenne*. The experimental design consisted of monocultures of *P. lanceolata* and mixtures of *L. perenne* and *P. lanceolata* exposed to three simulated climate scenarios. We hypothesized that (1) warming and elevated CO_2 alter foliar nutrients and defence molecules, (2) altered host quality and plant resistance affect insect herbivore performance, (3) warming and elevated CO_2 indirectly influence host quality and plant resistance via effects on neighbouring plants.

5.3 MATERIALS AND METHODS

5.3.1 Experimental set-up

The study was performed at the Drie Eiken Campus, University of Antwerp, Wilrijk, Belgium (51° 09' N, 04° 24'E) in 12 sunlit, south-facing, climate-controlled chambers. Details of this experimental platform can be found in Naudts *et al.* (2014). Three climate scenarios (four chambers per scenario) were simulated in an additive design: (1) current atmospheric CO_2 concentration and temperature (C); (2) future atmospheric CO_2 and current temperature (CO_2); and (3) future atmospheric CO_2 and temperature (TCO_2). Climate scenarios with elevated CO_2 had a target CO_2 concentration of 620 µmol mol^{-1} and future temperature chambers simulated a continuous 3 °C warming above fluctuating ambient temperatures. Climate manipulations were based on the IPCC-SRES B2-scenario prediction of moderate change for the year 2100 (IPCC, 2001).

The CO_2 concentration in each chamber was continuously measured and maintained at the target concentration with a CO_2 control group with an infrared analyser (WMA-4, PPSystems, Hitchin, UK). Air temperature and relative humidity were monitored every 0.5 h with a combined humidity–temperature sensor (Siemens QFA66, Erlangen, Germany), by averaging instantaneous readings in half hour mean values. During the experiment the CO_2 concentration was 382 ± 55 µmol mol^{-1} (SD) in the current climate, while it was 615 ± 70 µmol mol^{-1} (SD) in the climate scenarios with future CO_2 concentration (CO_2 and TCO_2). The monthly average air temperature in the C and CO_2 chambers was 16.2, 17.2 and 18.7 °C in June, July and August, respectively. TCO_2 chambers were 2.9 ± 1.0 °C (SD) warmer than current temperature chambers. Average vapour pressure deficit was 0.60 ± 0.34 and 0.64 ± 0.52 kPa (SD) in the climate treatments with ambient and warmed air, respectively. Irrigation was calculated as in Naudts *et al.* (2014). Total monthly irrigation equalled 64.4, 85.1 and 80.2 mm in June, July and August, respectively. Water freely drained while capillary rise was prevented by a drainage system placed below the chambers. The future climate chambers (TCO_2) received the same amount of water as the current climate chambers, so that any increase in water consumption would result in (aggravated) soil drought.

5.3.2 Plant and insect communities

We used two common co-occurring grassland species, *L. perenne* and *P. lanceolata*. *P. lanceolata* is characterized by the presence of the iridoid glycosides aucubin and catalpol. These compounds stimulate feeding and oviposition by specialist insects and can act as efficient defences against generalists herbivores (Bowers & Puttick, 1988; Puttick & Bowers, 1988). Both plant species were sown at the end of March in a non-climate controlled greenhouse with a time lag of one week to prevent size differences at the start of the experiment (Cotrufo & Gorissen, 1997), and were watered twice a week. Four or five week-old seedlings were transplanted into PVC containers (24 cm inner diameter and 40 cm height), filled with sandy soil (93.9% sand, 4.1% silt, 2.0% clay; pH 7.5; Kjeldahl-N 0.125 g kg^{-1}; 2.1% C in humus). Each of the 12 chambers received 20 containers with two different compositions: (1) 10 monocultures of *P. lanceolata*, and (2) 10 mixtures of both plant species in a 50:50 ratio. Each community contained 18 individuals planted in a hexagonal grid with 4.5 cm interspace. Interspecific interactions were maximized by avoiding clumping. All communities were fertilized with 10 g m^{-2} NH_4NO_3, 5 g m^{-2} P_2O_5, 10 g m^{-2} K_2O and micro-elements (Fe, Mn, Zn, Cu, B, Mo), given dissolved in water in two equal amounts.

When the seedlings were three months old, *P. lanceolata* was involuntarily infested with powdery mildew *Podosphaera plantaginis*. *P. plantaginis* is a biotrophic fungal pathogen which means that it feeds on living plant tissue but does not kill the infected host. Biotrophic parasites extract nutrients from living cells and have extended periods of physiological interaction with their hosts (Agrios, 2005). The powdery mildew was found in all climate scenarios and plant compositions. We took advantage of this unplanned infestation to study its putative additive effects on plant-insect herbivore interaction. Moreover, warming and elevated CO_2 have been shown to affect the severity of pathogen infections (Thomas & Blanford, 2003; Mikkelsen *et al.*, 2015).

The rosy apple aphid *D. plantaginea* was used as an insect herbivore. It overwinters as eggs on apple trees, the primary host plant, and migrates in spring to the obligate alternate hosts, *Plantago major* L. and *P. lanceolata* (Alford, 2014). On *Plantago* spp., they give birth to apterous (wingless)

morphs that reproduce by parthenogenesis (Blommers *et al.*, 2004). *L. perenne* is not a host plant for *D. plantaginea*. The aphids were reared in small cages on *P. lanceolata* under laboratory conditions of 22 ± 1 °C. They were introduced on 20-week old *P. lanceolata* seedlings. At this time, two adult, apterous aphids were placed with a dry paintbrush on the apex of each *P. lanceolata* plant in monocultures and mixtures. Consequently, at the start of the infestation each container contained 36 (monocultures) or 18 (mixtures) aphids. In each chamber, four monocultures of *P. lanceolata* and four mixtures were randomly chosen for aphid infestation. Containers that did not receive aphids acted as controls. During infestation, all containers (both control and herbivory treatments) were individually enclosed with a 85-cm-tall cylinder of lightweight netting to ensure aphids did not migrate between pots. The infrastructure did not physically limit plant growth and did not cause photosynthetic stress effects (F_v/F_m, the intrinsic efficiency of PSII in controls = 0.84 which is an optimal value (Johnson *et al.*, 1993)).

5.3.3 Data collection

In the fourth week after the aphid introduction we determined the mildew infestation and harvested the plants of two replicate communities per treatment in each chamber (totalling 2 replicate communities × 4 treatments × 12 chambers). The degree of powdery mildew on *P. lanceolata* was categorized by a rating system: 1) healthy (no visible lesions); 2) 1% - 25% of the leaves damaged; 3) 26% - 50% of the leaves damaged; 4) 51% - 75% of the leaves damaged; 5) greater than 75% of the leaves damaged. At the same time, aphid populations of four replicate communities per plant composition in each chamber were collected (totalling 4 replicate communities × 2 plant compositions × 12 chambers). Aphids were brushed directly into 70% ethanol with a dry paintbrush. It was not possible to collect all the aphids because their number per container was either too high or too low (hardly to find), therefore a subsample of the population per container was collected: we searched for and collected aphids for 30 minutes. The total number of aphids per community was divided by the number of *P. lanceolata* individuals in that community. During harvest, live aboveground plant biomass was separated from dead by species. Shoots were dried at 70 °C for 48 h and then weighed. For each community, the total aboveground plant biomass per species was divided by the number of that species'

individuals, providing us with primary data for our statistical analysis. The live aboveground biomass of *P. lanceolata* plants of the two harvested communities per treatment in each chamber (2 replicate communities × 4 treatments × 12 chambers) was ground in a mill, and three subsamples of each community were analyzed for nitrogen and carbon content using a NC element analyser (NC-2100 element analyser, Carlo Erba Instruments, Milano, Italy). The three subsamples were averaged prior to data analysis.

A separate subsample of the milled live aboveground biomass of *P. lanceolata* of the two harvested communities per treatment in each chamber (2 replicate communities × 4 treatments × 12 chambers) was taken to quantify several biochemical parameters: fructose, sucrose, glucose, total soluble sugars, starch, total proteins, lipids, phosphor, cellulose, tannin, lignin, catalpol, aucubin, total phenols, tocopherols, carotenoids, total antioxidant capacity (TAC), jasmonic acid, salicylic acid, malondialdehyde (MDA) and proline (see supplementary material section 1 for a detailed overview of the applied methodology).

5.3.4 Data analysis

We focused on *P. lanceolata* because this is the host plant for the aphid and the powdery mildew. In a first step we examined whether some of the metabolites showed a similar response to all the three treatments (aphid infestation, climate scenario and plant composition) by using a hierarchical clustering analysis. The metabolites were converted to Z-scores, normalizing the rate of change among the twelve conditions. Z-scores were calculated as $Z = (\boldsymbol{x} - \text{mean})/SD$, where ***x is*** mean level of the metabolite of the two replicates for each climate scenario × plant composition × aphid infestation combination**,** the mean represents the mean value of the metabolite across all three treatments (climate scenario, plant composition and aphid infestation), and SD is the standard deviation of the metabolite across all three treatments (climate scenario, plant composition and aphid infestation). The Z-scores of metabolites were subjected to a hierarchical clustering analysis with an Euclidean distance metric, and visualized as a heat map representation using Multi Experiment Viewer (Mev) version 4.8 (Saeed *et al.*, 2003). The obtained clusters were used in the structural equation models (see below).

In a second step, we fitted two piecewise structural equation models (SEM), which combine information from multiple separate linear models into a

single causal network (Shipley, 2009). The first SEM investigated the effect of aphid infestation, climate scenario, plant composition and mildew infestation on the metabolites and live aboveground biomass of *P. lanceolata*. In a second SEM, we separated the metabolites of *P. lanceolata* in control pots from those with aphids. This allowed us to test the effects of the metabolites on the aphid population, as well as quantitative effects of the aphid population on these metabolites. The response of the aphid population was measured as their number at the end of the infestation. The four clusters obtained by the hierarchical clustering analysis were used to divide the metabolites in separate groups. We standardized the metabolites, the live aboveground biomass of *P. lanceolata* and the number of aphids by converting to Z-scores to equalize variances (see above). To reduce the number of mildew categories, we rearranged degree of mildew in two categories: (1) no or mild mildew infection (category 1 - 2) (2) severe mildew infection (category 3 - 5).

Traditional SEM estimation methods assume that all variables follow a normal distribution and all observations are independent (Grace, 2006). In our analyses, we used piecewise SEM that allows fitting general linear mixed effect models that can incorporate random effects. Each mixed effects model was fitted using the "lme" function in the "nlme" package (version 3.1-128) in R. For each model, we fitted a random effect of chamber. The overall path model (the SEM) was fitted using the "piecewiseSEM" package (version 1.2.1) in R (Lefcheck, 2016). Goodness of fit was estimated using Shipley's test of d-seperation, which yields a Fisher's C statistic that is Chi-squared distributed (Shipley, 2009). If the resulting P-value > 0.05, then the SEM can be said to adequately reproduce the hypothesized causal network.

In a third step, multiple Permutational Multivariate Analysis of Variance (PERMANOVA; with Adonis function in R; (Anderson, 2001)) were performed to evaluate the main and interactive effects of aphid infestation, climate scenario and plant composition on the metabolites of *P. lanceolata*. Because mildew infestation did not have a significant effect on metabolites (see result SEM below), this treatment was excluded in the PERMANOVA analysis. The attributed chamber was always included as a random effect. This analysis tests to which degree Euclidean distances between and within treatments differ from random expectations. Because it is distribution-free, different measures following different distributions can be integrated into

one multivariate analysis. All measured responses were scaled to give them equal weight in the permutational analysis. It should be noted that the results derived from the PERMANOVA are occasionally different from those obtained with SEM, as the latter used averages of the metabolites in each cluster.

In a final step, all data were analyzed with General Linear Mixed models (GLM) in SAS (version 9.2, SAS Institute Inc., Cary, NC) (Littell *et al.*, 1996) with chamber as a random factor nested within climate scenario. Climate scenario, plant composition, aphid infestation and two-way and three-way interactions between these predictors were included as fixed factors. Because mildew infestation did not have a significant effect on biochemical plant responses, this treatment was excluded in the GLM analysis. Non-significant factors were backwards-excluded from the model. In case of significant effects, *a posteriori* means comparisons using Tukey test corrected for multiple comparisons were made. Effects were considered significant at $P \leq 0.05$.

5.4 RESULTS

5.4.1 Hierarchical clustering analysis of metabolites

The primary and secondary metabolites of *P. lanceolata* were subjected to a hierarchical clustering analysis on the basis of their response to aphid infestation, climate scenario and plant composition. Using a distance cut off of 0.3, we obtained four major clusters of metabolites (1 - 4 in Fig. 1). The first cluster contains the primary antioxidants total phenols and tocopherol, a membrane-embedded lipophilic molecule, in addition to the C:N ratio and tannins (Fig. 1). Tannins are commonly found metabolites with a high affinity for proteins, producing protein-tannin complexes which decrease the nutritional value of plant tissue (Schoonhoven *et al.*, 2005). Also the non-structural carbohydrates - total soluble sugars, glucose, fructose and starch - were classified in the first cluster. The second cluster contains another primary antioxidant molecule i.e. carotenoid, jasmonic acid, total proteins, P and lipids (Fig. 1). Jasmonic acid is an important plant hormone that controls plant defences against herbivores (Wu & Baldwin, 2010). The third cluster includes only the macronutrient N whereas the macronutrient C and the ratios C:P, N:P and lignin:N were classified in a fourth and last cluster (Fig. 1). This last cluster also contains metabolites that play an important role in plant defences against herbivores such as the plant hormone salicylic acid, iridoid glycosides (catalpol and aucubin), TAC, lignin, cellulose and proline. Also MDA, a commonly used indicator of membrane damage due to reactive oxygen species (ROS), and the soluble sugar sucrose were ordered in the fourth cluster. In conclusion, secondary metabolites of *P. lanceolata* were classified in different clusters, indicating different response to aphid infestation, climate scenario and plant composition.

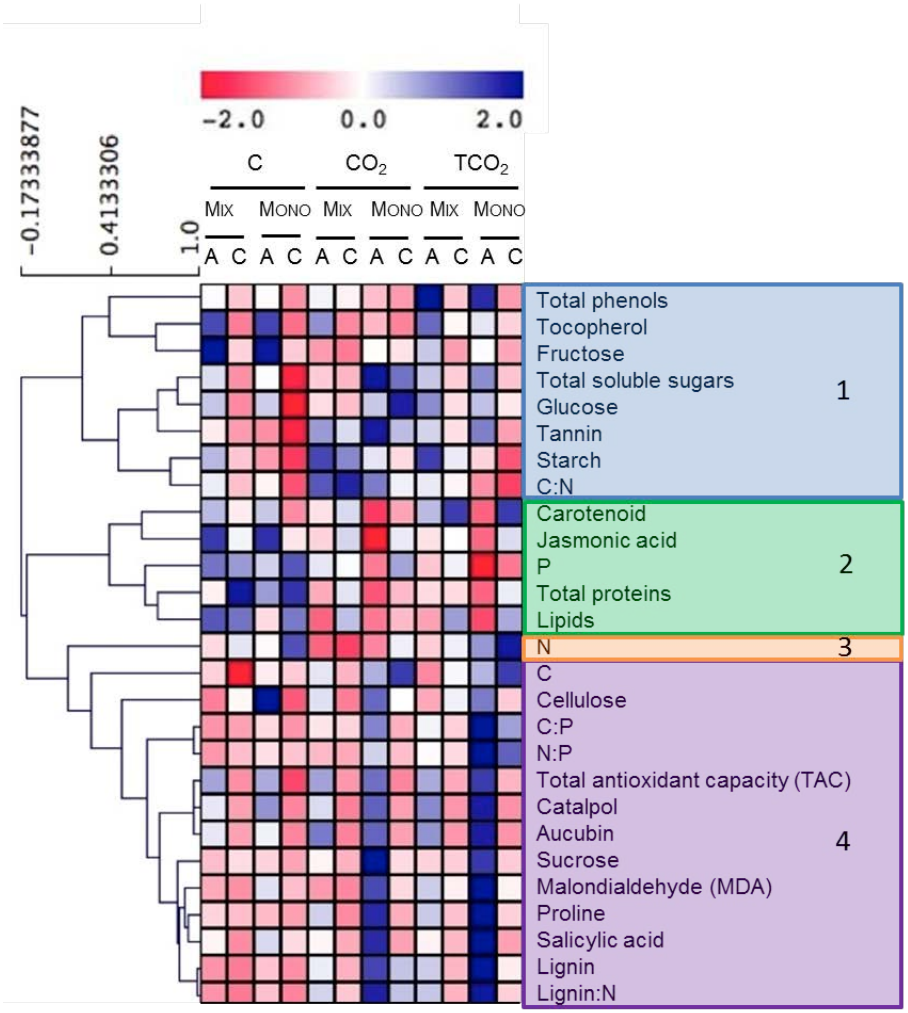

Fig. 1 Heat map showing the metabolite levels in the leaves of *P. lanceolata*, normalized to Z-score for each metabolite (blue-white-red heat map). Red and blue colours indicate a low and high metabolite level, respectively. Clustering was based on the Euclidean distance for metabolites. Labels 1-4 and colours indicate the four prominent clusters. Labels C, CO_2 and TCO_2 indicate current climate, elevated CO_2 and combined warming and elevated CO_2, respectively. Plant communities consist of monocultures of *P. lanceolata* (mono) and mixtures of *Lolium perenne* and *P. lanceolata* (mix) with (A) and without aphids (C).

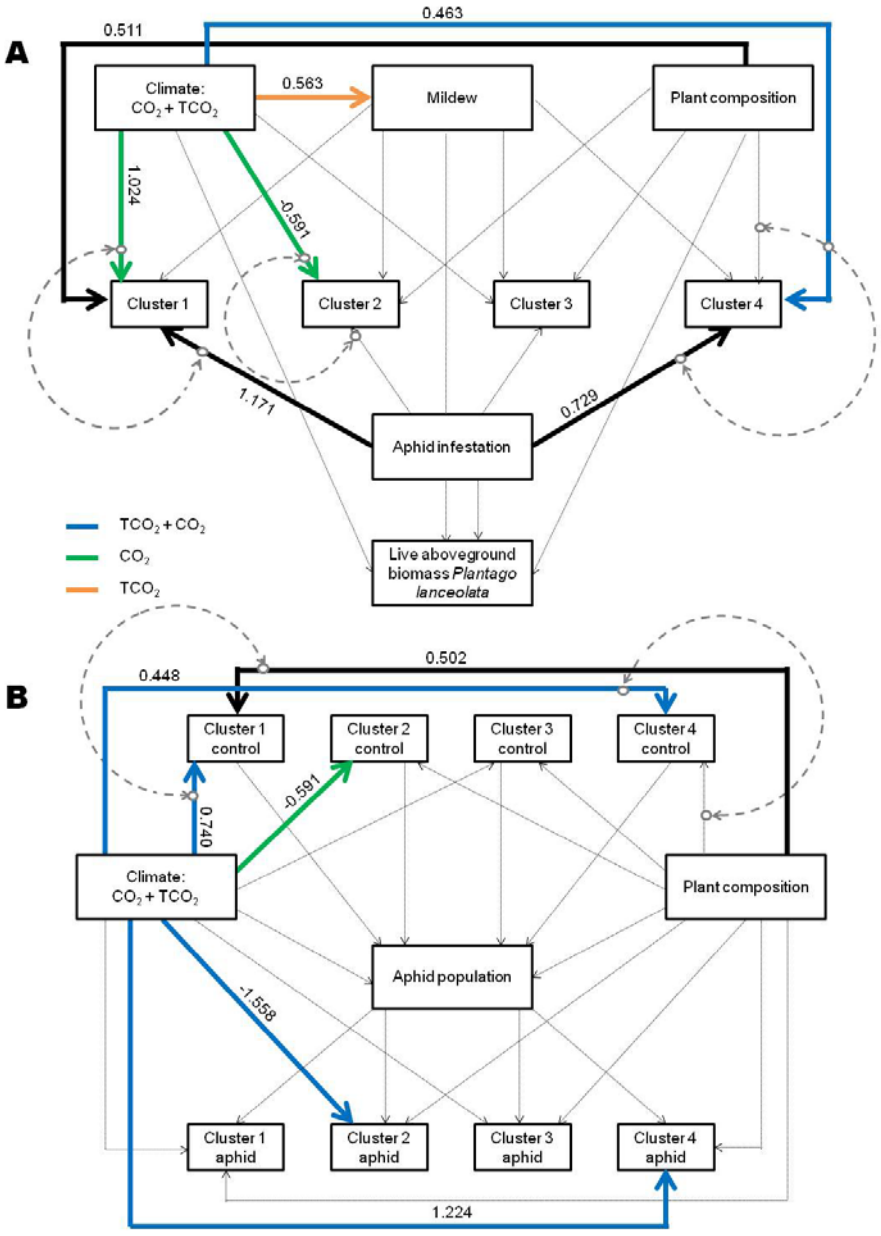
A
0.511
0.463
Climate:
$CO_2 + TCO_2$
0.563
Mildew
Plant composition
1.024
-0.591
Cluster 1
Cluster 2
Cluster 3
Cluster 4
1.171
0.729
Aphid infestation
$TCO_2 + CO_2$
CO_2
TCO_2
Live aboveground biomass *Plantago lanceolata*
B
0.448
0.502
Cluster 1 control
Cluster 2 control
Cluster 3 control
Cluster 4 control
-0.591
0.740
Climate:
$CO_2 + TCO_2$
Plant composition
Aphid population
-1.558
Cluster 1 aphid
Cluster 2 aphid
Cluster 3 aphid
Cluster 4 aphid
1.224

Fig. 2 (A) Structural equation model showing how climate scenario (CO_2 and TCO_2), mildew infestation, plant composition and aphid infestation affect the chemical composition and the live aboveground biomass of *P. lanceolata.* (B) Structural equation model showing how climate scenario, plant composition and chemical composition of *P. lanceolata* affect aphid population and how aphid population, in turn, affects the chemical composition of *P. lanceolata.* The four clusters refer to those obtained by the hierarchical clustering analysis (see Fig. 1). Solid black, green and orange arrows represent significant relationships ($P \leq 0.05$) and dashed grey lines significant interactions. Blue lines stand for significant effects of both CO_2 and TCO_2, green lines for significant effects of CO_2 and orange lines for significant effects of TCO_2. Light grey arrows represent nonsignificant relationships. Standardized path coefficients are shown next to pathways. For the effect of CO_2 and TCO_2, the average path coefficients are shown. The individual path coefficients of CO_2 and TCO_2 can be seen in Table S1 and Table S2 (see supplementary material section 2). Metabolites levels, live aboveground biomass of *P. lanceolata* and number of aphids were scaled before analysis.

5.4.2 Overview by SEM

The first SEM presents the effect of aphid infestation, climate scenario, plant composition and mildew infestation on the chemical composition and the live aboveground biomass of *P. lanceolata* (Fig. 2A). The metabolites of *P. lanceolata* were subdivided into four groups obtained from the hierarchical clustering analysis. The hypothesized structural relationship adequately fits the data (χ^2 = 42.53, df = 32, p = 0.101). Fig. 2A and Table S1 (see supplementary material section 2) provide us with three insights. Firstly, TCO_2 increased the mildew infestation compared to C but the mildew infestation, in turn, did not affect the metabolites and the live aboveground biomass of *P. lanceolata*. Secondly, neither climate scenario, nor aphid infestation or plant composition had a significant impact on the live aboveground biomass of *P. lanceolata*. Thirdly, the metabolites in *P. lanceolata* were differently affected by climate scenario, aphid infestation and plant composition. These three treatments increased the metabolites in cluster 1. Moreover, there was a significant interaction between climate scenario and aphid infestation. Also the metabolites in cluster 2 differed according to an interaction between climate scenario and aphid infestation. However, the three treatments did not affect the metabolites in cluster 3. Aphid infestation, CO_2 and TCO_2 increased the metabolites in cluster 4. Besides these treatments effects, climate scenario, aphid infestation and plant composition interacted with each other to affect the metabolites in cluster 4.

The second SEM presents the effect of climate scenario, plant composition and chemical composition of *P. lanceolata* on the aphid population and whether the aphid population, in turn, altered the chemical composition of *P. lanceolata*. The results of this SEM model show the following goodness of fit statistics: χ^2 = 77.52, df = 80 and p = 0.558. Fig. 2B and Table S2 (see supplementary material section 2) provide us with two insights. Firstly, climate scenario, plant composition and the chemical composition of *P. lanceolata* did not affect the number of aphids. Secondly, the aphid populations in turn, did not change the chemical composition of *P. lanceolata*. Bringing together the findings from both SEM models, we conclude that the treatment aphid infestation altered the chemical composition of *P. lanceolata* but the number of aphids had no effect on it.

5.4.3 Results from GLM analyses

Climate scenario, but not plant composition or aphid infestation altered the live aboveground biomass of *P. lanceolata* ($F_{2,9}$ = 6.34, p = 0.019; $F_{1.82}$ = 0.84, p = 0.362; $F_{1.82}$ = 0.98, p= 0.325, respectively, Fig. 3). The live aboveground biomass tended to be higher in CO_2 compared to C and was significantly lower in TCO_2 compared to CO_2. However, the biomass in TCO_2 was not significantly different from that of C. Plant composition, but not climate scenario or aphid infestation altered the dead aboveground biomass of *P. lanceolata* ($F_{1,75}$ = 72.82, p = <0.001; $F_{2.9}$ = 0.93, p = 0.428; $F_{1,75}$ = 0.71, p = 0.401, respectively, not shown). Competition between *P. lanceolata* and *L. perenne* reduced the dead aboveground biomass. Furthermore, climate scenario and plant composition did not alter relative herbivory effects ($F_{2.42}$ = 0.02, p = 0.981; $F_{1.42}$ = 2.37, p = 0.131, respectively, not shown). Relative herbivory effects were calculated as (live aboveground biomass of *P. lanceolata* with herbivores – live aboveground biomass of *P. lanceolata* without herbivores)/(live aboveground biomass of *P. lanceolata* without herbivores). In addition, climate scenario and plant composition did not alter the number of aphids per plant ($F_{2,9}$ = 0.45, p = 0.650; $F_{1,78}$ = 0.87, p = 0.353, respectively). Aphid number tended to increase at higher CO_2 levels (7.15 ± SE 1.98 in CO_2 and 9.41 ± SE 2.26 in TCO_2, versus 5.07 ± SE 1.63 in C), but these increases were not significant.

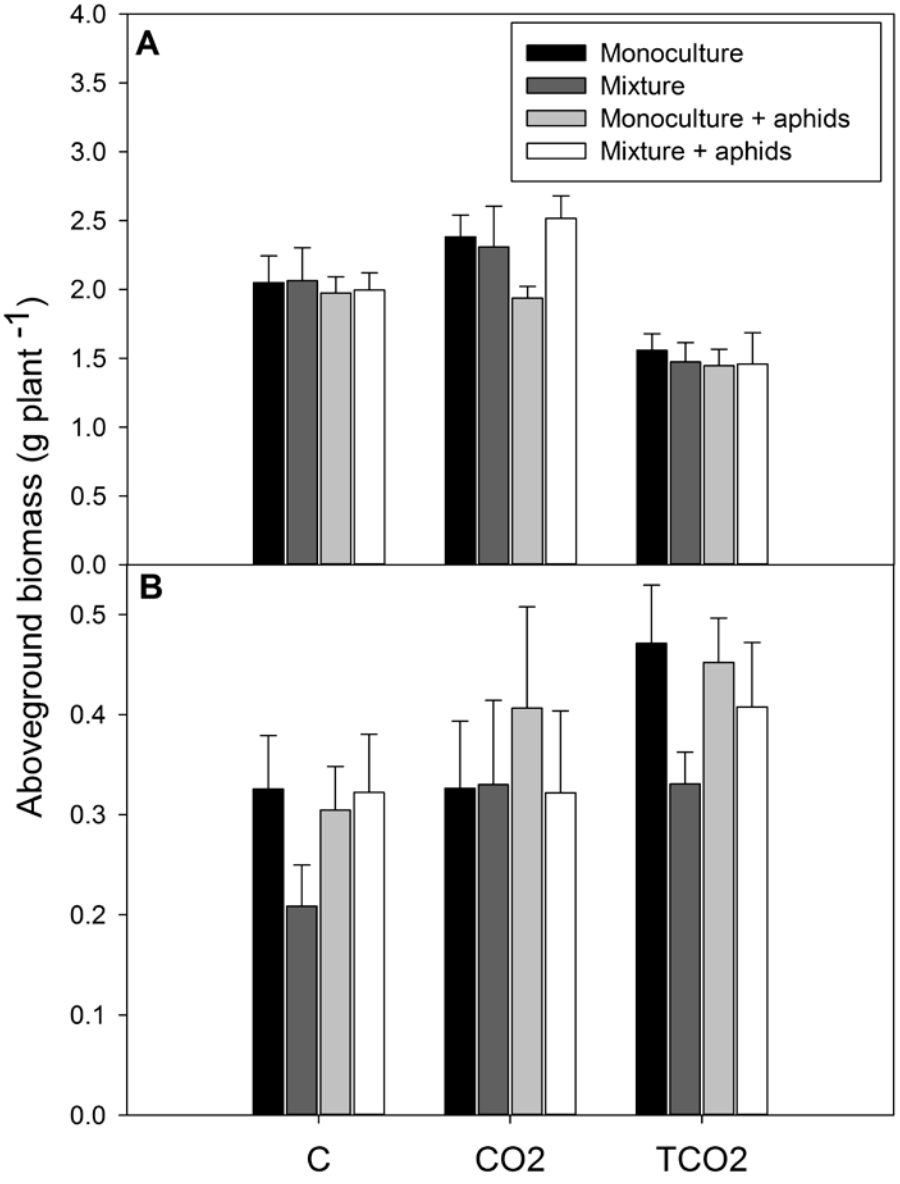

Fig. 3 Effect of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition on the live aboveground biomass (A) and the dead aboveground biomass (B) of *Plantago lanceolata*. Bars represent means ± SE. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata.*

A summary of GLM results for effects of climate scenario, plant composition and aphid infestation on the metabolites in *P. lanceolata* can be found in supplementary material section 3 and section 4 (Table S1 - S6 and Fig. S1 - S6).

As a baseline, the aphid infestation increased the concentrations of the metabolites in cluster 1 irrespective of climate scenario and plant composition (except for C:N ratio) (Table 1). However, the effect of aphid infestation on the concentrations of tannin depended on a significant interaction with plant composition. Aphid infestation increased the concentration of tannin in monocultures but not in mixtures. For glucose, fructose and total phenol, the effect of aphid infestation depended on a significant interaction with climate scenario. The concentration of glucose and fructose increased only with aphid infestation in C while the concentrations of total phenol increased with aphid infestation in C and TCO_2 (but not in CO_2). Moreover, TCO_2 strengthened the effect of aphid infestation on the concentration of total phenol. In addition, CO_2, compared to C, increased the concentrations of starch and the C:N ratio. TCO_2 in turn, reduced the concentration of starch (except for mixtures with aphids) and the C:N ratio compared to CO_2. The effect of climate scenario on the concentrations of soluble sugar, glucose and tannin depended on the plant composition. The concentrations of soluble sugars and glucose increased at elevated CO_2 but only in monocultures and TCO_2 did not alter the concentrations of glucose but decreased the concentrations of soluble sugars in monocultures. In general CO_2, compared to C, increased the concentration of tannin while TCO_2 did not alter it. Pairwise comparison revealed that in C the concentrations of tannins were higher in mixtures compared to monocultures while the opposite was true in CO_2. There was no difference between monocultures and mixture in TCO_2. Tocopherol varied according to a three-way interaction of aphid infestation, climate scenario and plant composition. Aphid infestation increased the concentration of tocopherol in monoculture and mixtures in C while in CO_2 and TCO_2 the increase under aphid infestation was more noticeable in mixtures. Furthermore, the concentration of tocopherol in monocultures with aphids declined at CO_2 compared to C but did not alter compared to TCO_2. Despite this, the concentrations were higher in mixtures without aphids at TCO_2 compared to CO_2 and C. Furthermore, interspecific interactions between *P. lanceolata*

and *L. perenne* increased the concentrations of total phenols, starch and the C:N ratio, but did not modify the concentrations of fructose.

The concentration of jasmonic acid, lipids and total proteins (cluster 2) varied according to a three-way interaction of climate scenario, plant composition and aphid infestation (Table 1, Fig. 4C). The concentration of jasmonic acid in C increased with aphid infestation in monocultures and mixtures but aphid infestation in CO_2 and TCO_2, decreased it in monocultures. Interspecific interactions between *P. lanceolata* and *L. perenne* mitigated the negative effect of aphid infestation in these climate scenarios. Furthermore, aphid infestation decreased the concentration of proteins in mixtures in C but did not alter the concentrations in monocultures and mixtures in CO_2 and TCO_2. In general, CO_2 compared to C decreased the concentration of proteins irrespective of the plant composition while aphid infestation and TCO_2, compared to CO_2 did not alter it. The concentration of lipids decreased with aphid infestation in monocultures and mixtures in all climate scenarios (except for mixtures in C). Also CO_2, compared to C, decreased the concentration of lipids in monocultures without aphid and mixtures with aphids but in TCO_2, compared to CO_2, this concentration increased again to similar levels as C in monocultures without aphids. For carotenoids, there was a significant interaction between aphid infestation and climate scenario. Aphid infestation in C did not alter the concentration of carotenoids while aphid infestation in TCO_2 reduced it. This is mainly due to an increase in the concentration of carotenoids in TCO_2 in controls, compared to C and CO_2. Aphid infestation reduced the concentration of P in monocultures but did not alter it in mixtures. Furthermore, also CO_2, compared to C, reduced the concentration of P and the concentration was further reduced in TCO_2.

Aphid infestation and interspecific plant interactions slightly reduced the leaf nitrogen content (cluster 3) (Table 1). However, climate scenario did not alter it. In should be noted that these results are different from those obtained by the SEM.

Aphid infestation, irrespective of the climate scenario considerably increased the concentration of the metabolites of cluster 4 (except carbon) but more in monocultures than in mixtures (Table 1, Fig. 4F). In other words, interspecific plant interactions between *P. lanceolata* and *L. perenne* mitigated the effect of aphid infestation. However, the concentration of

aucubin increased with aphid infestation in both monocultures and mixtures. Furthermore, the metabolites of cluster 4 (except cellulose, catalpol, aucubin, carbon and MDA) varied also according to a significant interaction between plant composition and climate scenario (Table 1). Elevated CO_2, compared to CO_2, increased the metabolites of cluster 4 but this positive effect of a future climate was mitigated by interspecific plant interaction (Table 1, Fig. 4D). TCO_2, compared to CO_2, increased further the N:P and the C:P ratio in monocultures but did not alter the concentration of lignin, sucrose, salicylic acid, proline, TAC, and the ratio lignin:N. Climate scenario did not alter the concentration of catalpol and carbon. For proline, lignin, cellulose and salicylic acid, there was a significant three-way interaction between climate scenario, plant composition and aphid infestation. CO_2 and TCO_2 strengthened (salicylic acid and lignin) or induced (proline) an effect of aphid infestation but only in monocultures. The concentrations of lignin and proline did not differ between CO_2 and TCO_2 while the concentration of salicylic acid increased in monocultures with aphids in TCO_2 compared to CO_2. For cellulose, the three-way interaction between climate scenario, plant composition and aphid infestation was mainly due to a significant increase in the concentration of cellulose in monocultures with aphids in C compared to other treatments in C, mixtures without aphids in CO_2 and mixtures with aphids in TCO_2. In addition, CO_2 and TCO_2 also strengthened the effect of aphid infestation on the concentration of aucubin but irrespective of the plant composition. Furthermore, TCO_2 increased the concentration of MDA compared to the other climate scenarios and this was most clear in monocultures with aphids.

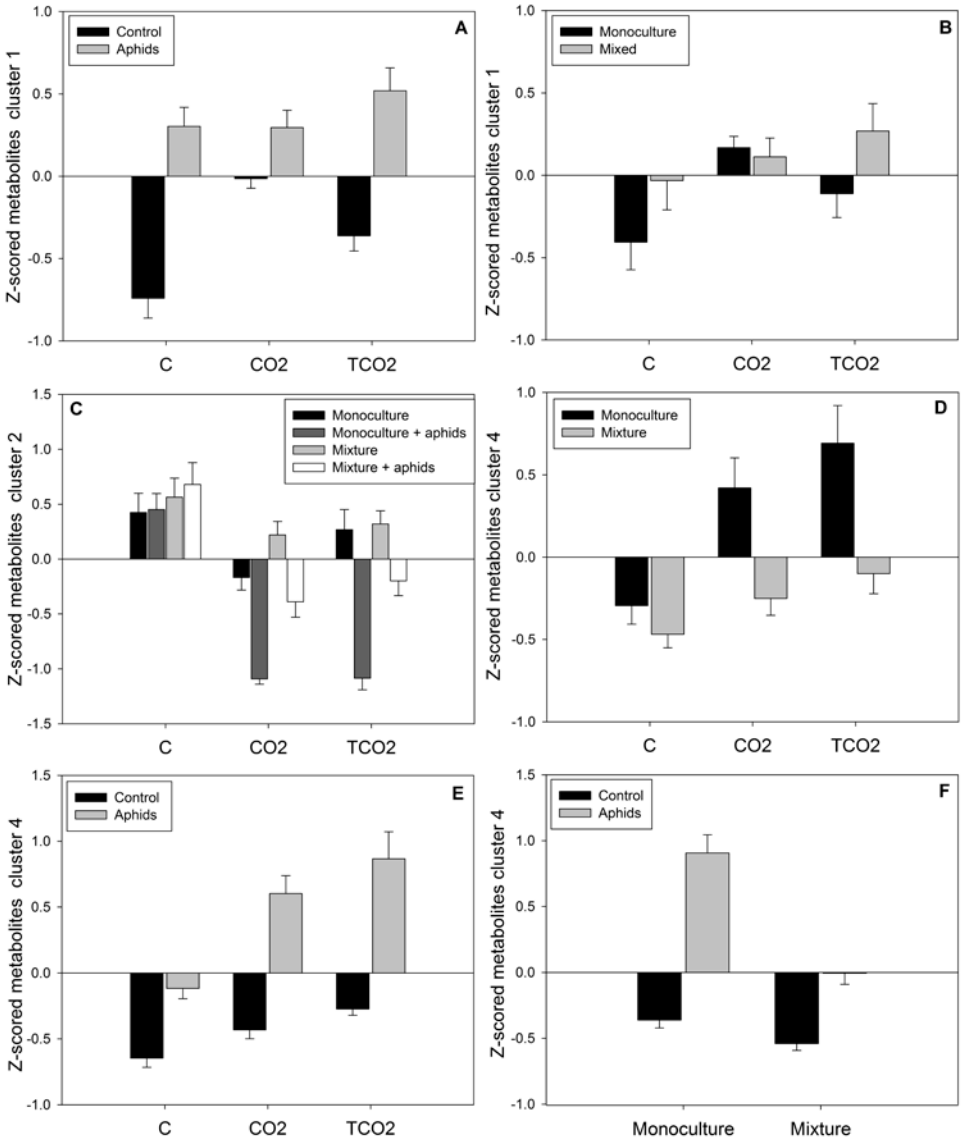
A
Control
Aphids
Z-scored metabolites cluster 1
1.0
0.5
0.0
-0.5
-1.0
C
CO2
TCO2
B
Monoculture
Mixed
Z-scored metabolites cluster 1
C
Monoculture
Monoculture + aphids
Mixture
Mixture + aphids
Z-scored metabolites cluster 2
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
D
Monoculture
Mixture
Z-scored metabolites cluster 4
E
Control
Aphids
Z-scored metabolites cluster 4
F
Control
Aphids
Z-scored metabolites cluster 4
Monoculture
Mixture

Fig. 4 Interactive effects of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition (monocultures and mixtures) on the metabolite levels of *Plantago lanceolata*. (A) Effect of climate scenario and aphid infestation on the metabolite levels in cluster 1. (B) Effect of climate scenario and plant composition on the metabolite levels in cluster 1. (C) Effect of climate scenario, aphid infestation and plant composition on the metabolite levels in cluster 2. (D) Effect of climate scenario and plant composition on the metabolite levels in cluster 4. (E) Effect of climate scenario and aphid infestation on the metabolite levels in cluster 4. (F) Effect of plant composition and aphid infestation on metabolite levels in cluster 4. Metabolites levels were normalized to Z-score for each metabolite. Bars represent the average metabolite level ± SE. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*.

Table 1 Summary of PERMANOVA results for effects of climate scenario, plant composition and aphid infestation on the metabolite levels of *P. lanceolata.* Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*. P-values are presented in bold when significant (≤0.05).

	Treatment	df	F	P
Cluster 1	Climate scenario	2	13.292	**0.001**
	Plant composition	1	14.098	**0.001**
	Aphid infestation	1	35.353	**0.001**
	Climate scenario× plant composition	2	3.785	**0.004**
	Climate scenario× aphid infestation	2	6.747	**0.001**
	Plant composition × aphid infestation	1	1.628	0.165
	Climate scenario × aphid infestation × plant composition	2	0.897	0.518
Cluster 2	Climate scenario	2	23.687	**0.001**
	Plant composition	1	11.896	**0.001**
	Aphid infestation	1	23.971	**0.001**
	Climate scenario× plant composition	2	1.908	0.095
	Climate scenario× aphid infestation	2	11.599	**0.001**
	Plant composition × aphid infestation	1	2.388	0.068
	Climate scenario × aphid infestation × plant composition	2	3.931	**0.005**

	Treatment	df	F	P
Cluster 3	Climate scenario	2	2.89	0.108
	Plant composition	1	9.82	**0.003**
	Aphid infestation	1	4.74	**0.033**
	Climate scenario× plant composition	2	0.49	0.615
	Climate scenario× aphid infestation	2	0.25	0.780
	Plant composition × aphid infestation	1	1.95	0.167
	Climate scenario × aphid infestation × plant composition	2	0.15	0.857
Cluster 4	Climate scenario	2	17.378	**0.001**
	Plant composition	1	27.864	**0.001**
	Aphid infestation	1	86.838	**0.001**
	Climate scenario× plant composition	2	4.750	**0.003**
	Climate scenario× aphid infestation	2	4.848	**0.003**
	Plant composition × aphid infestation	1	13.083	**0.001**
	Climate scenario × aphid infestation × plant composition	2	2.077	0.082

5.5 DISCUSSION

In this study, we investigated not only the individual effect of elevated CO_2, but also the combined effect of elevated CO_2 and warming, on host quality for herbivores and plant defence compounds. We examined if and how both these attributes influence insect herbivore performance. TCO_2 modified the leaf quality of *P. lanceolata*. CO_2 enhanced both the induced and systemic defence system, as this treatment increased specific defence molecules in *P. lanceolata* plants with and without aphids. These effects of elevated CO_2 were not modified by warming. Surprisingly, interspecific plant interactions neutralized the positive effect of elevated CO_2 on some of the defence molecules. Although CO_2 and TCO_2 altered the leaf quality and defence molecules, we found no indications of indirect effects on aphid performance.

5.5.1 *Plantago lanceolata* showed induced direct resistance against its herbivore in the current climate

As expected, aphid infestation reduced N, P, total proteins and lipids in *P. lanceolata*. These nutrients are part of the nutritional requirements of insect herbivores (Schoonhoven *et al.*, 2005). Furthermore, aphid feeding induced the chemical defence molecules catalpol, aucubin, tannin, lignin and cellulose. Previous studies have found contradictory results regarding the inducibility of iridoid glycosides (catalpol and aucubin) in *P. lanceolata*, either finding induction after herbivory (Bowers & Stamp, 1993; Darrow & Bowers, 1999) or not (Stamp & Bowers, 1996; Jarzomski *et al.*, 2000). We provide support for induced response of these compounds in *P. lanceolata*. In contrast to previous research, aphid infestation led to an increase in salicylic acid and jasmonic acid (Walling, 2000). It is often postulated that the salicylic acid pathway is turned on if plants are attacked by piercing-sucking insects such as aphids, whereas the jasmonic acid pathway is activated in response to chewing/biting herbivores (Walling, 2000; Kaloshian & Walling, 2005). However, more recent research provides evidence that attack by aphids also activates the jasmonic acid pathway (Kuśnierczyk *et al.*, 2011; Morkunas & Gabryś, 2011).

As a result of herbivory stress, the levels of ROS increase in plants (Wu & Baldwin, 2010). In our study, the induction of oxidative stress was assessed

by measuring MDA, which aphid herbivory considerably increased. A common defence response of plants against oxidative stress entails changes in ROS scavenging antioxidant metabolites. Indeed, TAC and the primary antioxidant molecules total phenol and tocopherols increased due to aphid herbivory. Moreover, *P. lanceolata* responded to herbivory by increasing non-structural carbohydrates including starch and soluble sugars. Also sugars can act as ROS scavengers or can have a signalling function in regulating stress and defence responses (Gómez-Ariza *et al.*, 2007; Peshev *et al.*, 2013). In addition, aphid infestation did not alter the live and dead biomass of *P. lanceolata* which means that induced resistance against oxidative stress and herbivory in *P. lanceolata* neutralised the biomass lost.

5.5.2 Elevated CO_2 and TCO_2 altered the biomass of *Plantago lanceolata* but not herbivory rates

Elevated CO_2 tended to increase the live biomass of *P. lanceolata* whereas TCO_2 tended to reduce it, compared to the current climate. Elevated CO_2 stimulates biomass production directly through enhanced photosynthesis, or indirectly through its effect on the hydrological cycle as elevated CO_2 decreases stomatal conductance, leading to increased water use efficiency (Long *et al.*, 2004; Dieleman *et al.*, 2012). Our result that warming significantly reduced the positive effect of elevated CO_2 on the biomass supports findings in other multifactorial studies (e.g. Lilley *et al.*, 2001; Williams *et al.*, 2007). High summer temperatures around the optimum temperature, can retard plant biomass production via associated drought and heat stress (Rawson, 1992; De Valpine & Harte, 2001; De Boeck *et al.*, 2008).

Elevated CO_2 and TCO_2 did not induce herbivory effects on the live biomass of *P. lanceolata*. Consequently, the relative biomass losses of *P. lanceolata* did not change significantly under CO_2 and TCO_2. This implies that, as found under strict laboratory conditions (Van De Velde *et al.*, 2016b), warming and CO_2 did not affect the net interaction strength between plants and herbivores.

5.5.3 Elevated CO_2 reduced the leaf quality and TCO_2 altered it

Elevated CO_2 increased starch, soluble sugars (in monocultures) and glucose (in monocultures) and lowered leaf proteins (except in mixtures with aphids), hereby increasing the C:N ratio. Reduced N-based metabolites at elevated CO_2, like proteins, is generally attributed to dilution by increased non-structural carbohydrates (Stiling & Cornelissen, 2007; Robinson *et al.*, 2012). In addition, elevated CO_2 reduced P and consequently increased the C:P ratio (in monocultures). As P and proteins are part of the nutritional requirements of insect herbivores (see above), their reduction implies a reduced nutritive value to herbivores (Mattson, 1980; Lincoln *et al.*, 1986; Schoonhoven *et al.*, 2005; Huberty & Denno, 2006).

Compared to CO_2, TCO_2 reduced the positive effect of CO_2 on starch (except in mixtures with aphids) and soluble sugars (in monocultures) to intermediate levels between C and CO_2, which lowered the C:N ratio. Furthermore, compared to CO_2, TCO_2 further decreased P, hereby increasing the C:P ratio but it did not alter leaf proteins. Consequently, TCO_2 altered the CO_2-induced reduction in leaf quality but the general direction is unclear as the C:N and the C:P ratio showed contrasting responses. Murray *et al.* (2013) reported that warming may alleviated the CO_2 reduction in leaf quality. However, an accurate comparison is not possible as they did not measure P concentrations.

5.5.4 Elevated CO_2 enhanced both systemic and induced defence but warming did not alter these effects of elevated CO_2

Elevated CO_2 increased the defence molecules lignin and tannin but did not alter catalpol and cellulose. Remarkably, CO_2 not only influenced these defence molecules but also enhanced the effect that aphid infestation had on lignin and aucubin (i.e. significant climate scenario × aphid infestation interactions). Therefore, in line with previous research elevated CO_2 not only enhanced systemic defence but also influenced the inducibility of plant chemical defence (Bidart-Bouzat *et al.*, 2005; Bidart-Bouzat & Imeh-Nathaniel, 2008). However, in this study this was not straightforward for all defence molecules. Enhanced induced defence under elevated CO_2 may have

important implications for plant-insect herbivore interaction as induced defence can decrease herbivore damage, and consequently, improve plant fitness (Agrawal, 1999).

According to the carbon-nutrient-balance hypothesis, defence molecules should increase under CO_2 as result of the 'excess' carbon (Bryant *et al.*, 1983). Despite the higher C:N ratio under CO_2 in our study, the variable response of the secondary metabolites suggests that the C:N ratio does not regulate the way plants allocate resources between growth and secondary metabolism. Also previous research has shown that the carbon-nutrient-balance hypothesis fails as predictive hypothesis (Hamilton *et al.*, 2001; Lindroth, 2012). New studies have proposed that resource utilisation for chemical defence is linked with photosynthesis, hormone regulation and the control of gene expression (Zavala *et al.*, 2017). In particular, elevated CO_2 induces changes in plant hormones responsible for regulation of constitutive and enemy-induced secondary chemistry (Zavala *et al.*, 2013). Indeed, we found that CO_2 altered the induced synthesis of salicylic acid and jasmonic acid, phytohormones that play an important role in promoting compounds responsible for herbivore defence (Wu & Baldwin, 2010). Elevated CO_2 stimulated the synthesis of salicylic acid but suppressed the concentration of jasmonic acid, compared to the current climate but only in the presence of aphids. This result is analogous to previous studies in the plant families Brassicaceae and Solanaceae (Zavala *et al.*, 2013), and may thus be a general response.

Besides CO_2, also TCO_2 increased the defence molecules tannin, aucubin and lignin, while not altering catalpol and cellulose. Therefore, also TCO_2 enhanced both the systemic (tannin and lignin) and induced (aucubin and lignin) defence system of *P. lanceolata*. These effects of TCO_2 were clearly due to CO_2 as they did not differ from those of the CO_2 treatment. By contrast, in a meta-analysis, Zvereva & Kozlov (2006) showed that, simultaneous elevation of temperature and CO_2 did not alter phenolics (such as tannins and lignin), while warming increased terpenes (such as aucubin and catalpol) under elevated CO_2.

TCO_2 increased MDA which was mainly due to a positive effect of TCO_2 on the induced response in monocultures. Consequently, the induction of oxidative stress due to aphid herbivory may be higher in TCO_2 than in the current climate. Despite this, the different primary antioxidants molecules

showed an opposite response. Proline and total phenol increased in TCO_2 under aphid infestation while carotenoids and tocopherol decreased.

5.5.5 *Plantago lanceolata* – climate – herbivory interactions are mediated by interspecific plant competition

Interspecific plant interactions between *P. lanceolata* and *L. perenne* mediated the induced response of some metabolites in *P. lanceolata* in the current climate and the induced and systemic response in a future climate. Interspecific plant interactions mitigated the reduction of P and lipids and the increase of the defence molecules lignin, catalpol and cellulose and salicylic acid due to aphid herbivory. Several studies have shown that the presence and identity of neighbouring plants can influence the quality of the host plant by altering primary and secondary chemistry (Barton & Bowers, 2006; Broz *et al.*, 2010; Thorpe *et al.*, 2011; Lankau, 2012). These effects have often been attributed to growth-defence-trade-offs, a resource trade-off between the plant growth response to light competition and defence (Herms & Mattson, 1992). However, recent studies have shown that the down-regulation of plant defences under the influence of competition is directly mediated by light signals (Ballaré, 2014; Campos *et al.*, 2016), most notably the ratio of red to far-red light (Ballaré *et al.*, 1990). A reduced red to far-red ratio leads to a negative regulation of defences by a simultaneous inhibition of the jasmonic acid and the salicylic acid pathway (Wit *et al.*, 2013). In our study the levels of defence molecules and salicylic acid in *P. lanceolata* were higher when they were grown with conspecific neighbours than with heterospecific neighbours. This means that in our study, *P. lanceolata* may have been more constrained by light in interspecific plant interactions than in intraspecifc interactions. However, the down-regulation of plant defence against insect herbivores upon competition for light is a complex mechanism and includes more than just a resource trade-off (de Vries *et al.*, 2017).

CO_2 increased the difference in the concentrations of primary (P, sucrose, soluble sugars) and secondary metabolites (lignin, tannin, salicylic acid, jasmonic acid) between monocultures and mixtures. Interspecific plant interactions not only neutralized the positive effect of aphid infestation but also the positive effect of CO_2 on the defence system of *P. lanceolata*. Plant-

plant interaction thus adds a layer of complexity in mechanistic studies of climate change effects on herbivory.

5.5.6 Climate effects on aphid performance are limited

In general, CO_2 and TCO_2 did not significantly affect aphid abundance, though the aphid abundance tended to be larger under these treatments. Insect performance is thought to decrease under CO_2 due to the reduced nutritive value of the host plant (Robinson *et al.*, 2012). However, the responses to CO_2 have been shown to depend on the feeding guilds (Bezemer & Jones, 1998; Stiling & Cornelissen, 2007; Robinson *et al.*, 2012). Our result is consistent with a recent meta-analysis showing that, on average, the abundance of phloem feeders, such as aphids, increases, whereas foliage feeders respond negatively (Robinson *et al.*, 2012). Elevated CO_2 is thought to affect the aphids indirectly, through altered leaf quality. The quality of leaf tissue for insect herbivores depends on the concentration of essential nutrients (such as P and N) and on the concentration of defensive secondary compounds. Our structural equation model showed that these metabolites did not affect the aphid populations. This lack of association could be due to an inadequate change in the concentrations of the defence molecules. The mechanistic basis of a potentially positive effect of a future climate is likely driven by higher concentrations of essential amino acids in phloem (Ryan *et al.*, 2015). We did, however, not measure metabolite concentrations in the phloem sap. In addition, no direct effects of CO_2 and warming on aphids performance were observed.

5.5.7 Conclusion

Our semi-natural experimental approach demonstrated that aphid herbivory induced resistance against oxidative stress and herbivory in *P. lanceolata*, thereby neutralising loss of biomass. Elevated CO_2 increased specific defence molecules and enhanced the effect of aphid herbivory on these defence molecules. However, warming did not alter these effects of CO_2. Furthermore, CO_2 reduced the leaf quality for insect herbivores and TCO_2 altered the CO_2-induced reduction in leaf quality. Notwithstanding the effects of a future climate on leaf quality and defence molecules, we found no indications for indirect effects on aphid performances and herbivory. It is also possible that altered concentrations of essential amino acids in phloem

sap may have caused indirect effects on aphid abundance. As found under strict laboratory conditions (Van De Velde *et al.*, 2016b), warming and CO_2 affect several components of plant-herbivore interactions but were not found to change the net-interaction strength between plants and herbivores. Remarkably, we showed that plant-plant interactions altered the effect of a future climate on the primary and secondary metabolites. Therefore, we emphasise the need for community-scale experiments for a more thorough understanding of the effects of a future climate on vegetation dynamics.

5.6 ACKNOWLEDGEMENTS

H. Van De Velde is a Research Assistant of the Fund for Scientific Research-Flanders (FWO). D. Bonte was funded by the FWO research network "An eco-evolutionary network of biotic interactions". We thank the Earth and Life Institute (Université Catholique de Louvain) for providing *Dysaphis plantaginea*, N. Calluy and M. Wellens for technical assistance and colleagues for field assistance.

5.7 SUPPLEMENTARY MATERIAL

5.7.1 Section 1: material and methods

5.7.1.1 Carbohydrates

Small soluble sugars were determined in 0.2 g (DW) plant material, ground in liquid nitrogen (MagNALyser, Roche, Vilvoorde, Belgium) and extracted in 1 ml of 50 mM TAE buffer pH 7.5 (0.02% sodium azide, 10 mM mannitol, 0.1% polyclar, 10 mM $NaHSO_3$, 1 mM mercapto-ethanol, 1 mM phenylmethanesulfonylfluoride (PMSF)). The extract was centrifuged (14,000 g, 4 °C, 5 min), 150 µl was heated for 5 min in a water bath at 90 °C. After cooling and centrifugation (14,000 g, 4 °C, 5 min), the supernatant was added to a mixed bed Dowex column (300 µl Dowex H+, 300 µl Dowex Ac–; both 100–200 mesh; Acros Organics, Morris Plains, NJ, USA). The column was eluted six times with 150 µl of ddH2O. Glucose, fructose and sucrose concentrations were measured by HPAEC-PAD as before (Vergauwen *et al.*, 2000). Total soluble sugars and starch content were estimated by the anthrone reagent method (Leyva *et al.*, 2008).

5.7.1.2 Total protein

A plant sample (200 mg DW) was homogenized in 2 ml of cold 0.05M K-Phosphate buffer (pH7.0) and centrifuged at 15,000 g at 4 °C for 20 min. The supernatant was treated by 10% (w/v) TCA to precipitate soluble protein, which was redissolved in 1 N NaOH. The remaining pellet was used to extract insoluble protein. It was successively washed with 80% ethanol, 10% (w/v) cold TCA, ethanol:chloroform (3:1, v/v), ethanol:ether (3:1, v/v), and ether to remove phenolic compounds. The washed pellet was then dissolved in 1 N NaOH at 80 °C for 1 h. Soluble and insoluble protein content was estimated according to Lowry *et al.* (1951). Total protein content was calculated by adding the contents of soluble and insoluble proteins.

5.7.1.3 Lignin, polyphenols and tannin

For lignin determination, MagNALyser homogenized 0.1 g DW with 95% ethanol. The homogenate was centrifuged at 14 g for 3 min. Successively, the pellet was washed with 95% ethanol 30 min at 76 °C, chloroform for 30

min at 59 °C and then incubated in acetone for 30 min at 54 °C. One ml of 25% acetyl bromide in acetic acid (1:3, v/v) was added to the pellet and incubated at 70 °C for 30 min. After cooling, 0.2 ml of 2 M NaOH and 0.1 ml of 7.5 M hydroxylamine hydrochloride were added, and the volume was made up to 10 ml with acetic acid. After centrifugation at 1000 g for 5 min, the absorbance of the supernatant was measured against a NaOH blank at 280 nm (Lin & Kao, 2001). Polyphenol contents were extracted in 80% ethanol (v/v) and determined according to Zhang *et al.* (2006), with gallic acid as standard. Tannin content was determined as described by Hagerman and Butler (1978). About 0.2 g FW was homogenised in 2 ml acetate buffer pH 5 containing 2 mg of bovine serum albumin. The mixture was incubated for 15 min at room temperature and then centrifuged at 14,000 g for 15 min then the pellet was dissolved in 4 ml of a solution consisting of 1% SDS and 5% of tri-ethanolamine in water. One ml of 10 mM FeCl3 in 0.01 N HCl was added and incubated for 15 min. Then the absorbance was determined at 510 nm. Tannic acid was used as the standard.

5.7.1.4 Membrane damage (lipid peroxidation (MDA))

Lipid peroxidation was determined on 200 mg dry tissues, homogenized in 2 ml 80% ethanol by mortar and pestle, using a thiobarbituric acid-malondialdehyde (TBA-MDA) assay (Hodges *et al.*, 1999).

5.7.1.5 Total antioxidant capacity

Plant tissues (200 mg DW) were ground by a MagNALyser in liquid nitrogen and the antioxidants were extracted in 2 ml of ice cold 80% ethanol. FRAP (ferric reducing/antioxidant power assay) reagent (0.3 M acetate buffer (pH3.6), 0.01 mM TPTZ in 0.04 mM HCl, 0.02 M FeCl3.6H2O) was mixed with the extract and measured at 600 nm using a microplate reader (Synergy Mx, Biotek Instruments Inc., Vermont, USA) (Benzie & Strain, 1999). Trolox was used as standard.

5.7.1.6 Tocopherols

Tocopherols were extracted with hexane using the MagNALyser. The dried extract (CentriVap concentrator, Labconco, Kansas, USA) was resuspended in hexane, and tocopherols were separated and quantified by HPLC (Shimadzu, ‘s Hertogenbosch, The Netherlands) (normal phase conditions,

Particil Pac 5 µm column material, length 250 mm, i.d. 4.6 mm). Dimethyl tocol (DMT) was used as internal standard (5 ppm). Data were analysed with Shimadzu Class VP 6.14 software.

5.7.1.7 Catalpol and aucubin

Each sample was extracted overnight in 70 % methanol, and then filtered (12–15 µm) followed by a dilution of 10 times with ultrapure water. The concentrations of the aucubin and catalpol were analyzed using HPLC as described by Marak *et al.* (2002).

5.7.1.8 Salicylic acid and jasmonic acid

Salicylic acid concentration was measured according Li *et al.* (1999). This procedure had a 25% recovery rate, as determined by extracting known amounts of salicylic acid. Samples were homogenized in the extraction buffer. The samples were analysed by GC-MS after addition of 150 ng of 13C1,2-JA as an internal standard (Schittko *et al.*, 2000). The tissue was homogenized with a reciprocating shaker at 6.0 m sec ± 1 for 90 sec in extraction tubes containing 900 mg of lysing matrix (BIO 101, Vista, California, USA).

5.7.2 Section 2: supplementary tables: results from SEM analyses

Table S1 Partial slopes of the structural equation model presented in Figure 2A. Labels CO_2 and TCO_2 indicate elevated CO_2 and combined warming and elevated CO_2, respectively. Plant communities consist of monocultures of *Plantago lanceolata* and mixtures of *P. lanceolata* and *Lolium perenne*. The four clusters refer to those obtained by the hierarchical clustering analysis (see Fig. 1). P values are presented in bold when significant (≤0.05).

Response	Predictor	Estimate	SE	P-value
Mildew	TCO_2	0.563	0.232	**0.038**
	CO_2	-0.125	0.232	0.603
Cluster 1	Aphid infestation	1.171	0.217	**0.000**
	CO_2	1.024	0.224	**0.001**
	CO_2 × aphid infestation	-0.899	0.306	**0.007**
	Plant composition	0.511	0.219	**0.028**
	CO_2 × plant composition	-0.586	0.308	0.068
	TCO_2	0.438	0.233	0.093
	TCO_2 × aphid infestation	-0.324	0.306	0.300
	Plant composition × aphid infestation	-0.264	0.308	0.399
	TCO_2 × plant composition × aphid infestation	0.330	0.434	0.454
	CO_2 × plant composition × aphid infestation	0.329	0.433	0.454

Response	Predictor	Estimate	SE	P-value
	TCO_2 × plant composition	-0.163	0.308	0.600
	Mildew	0.036	0.129	0.780
Cluster 2	TCO_2 × aphid infestation	-1.382	0.323	**0.000**
	CO_2 × aphid infestation	-0.952	0.323	**0.007**
	CO_2	-0.591	0.229	**0.030**
	TCO_2 × plant composition × aphid infestation	0.746	0.459	0.116
	CO_2 × plant composition	0.244	0.325	0.459
	TCO_2	-0.151	0.237	0.541
	Plant composition	0.138	0.231	0.555
	CO_2 × plant composition × aphid infestation	0.228	0.457	0.622
	Plant composition × aphid infestation	0.090	0.325	0.785
	TCO_2 × plant composition	-0.086	0.325	0.793
	Aphid infestation	0.028	0.229	0.905
	Mildew	-0.010	0.127	0.939
Cluster 3	Aphid infestation	-0.748	0.397	0.071
	Plant composition	-0.549	0.403	0.185
	CO_2	-0.646	0.483	0.213

Response	Predictor	Estimate	SE	P-value
	Plant composition × aphid infestation	0.432	0.566	0.453
	Mildew	0.198	0.282	0.489
	TCO_2 × plant composition	-0.346	0.566	0.547
	TCO_2 × aphid infestation	0.214	0.561	0.707
	CO_2 × plant composition	-0.210	0.566	0.714
	CO_2 × aphid infestation	0.174	0.561	0.759
	CO_2 × plant composition × aphid infestation	0.239	0.794	0.766
	TCO_2	0.151	0.503	0.771
	TCO_2 × plant composition × aphid infestation	-0.182	0.797	0.821
Cluster 4	Aphid infestation	0.729	0.154	0.000
	TCO_2 × aphid infestation	0.987	0.218	0.000
	CO2 × aphid infestation	0.631	0.218	0.008
	TCO_2	0.525	0.160	0.010
	TCO_2 × plant composition × aphid infestation	-0.766	0.309	0.020
	CO_2	0.401	0.154	0.029
	CO_2 × plant composition	-0.388	0.219	0.089
	Plant composition × aphid infestation	-0.385	0.219	0.091

Response	Predictor	Estimate	SE	P-value
	TCO_2 × plant composition	-0.227	0.219	0.310
	CO_2 × plant composition × aphid infestation	-0.251	0.309	0.423
	Mildew	-0.062	0.086	0.472
	Plant composition	0.011	0.156	0.944
Biomass *P. lanceolata*	CO_2 × plant composition × aphid infestation	1.088	0.571	0.068
	TCO_2	-0.904	0.488	0.097
	CO_2 × aphid infestation	-0.626	0.404	0.133
	CO_2	0.566	0.474	0.263
	Mildew	0.142	0.229	0.542
	TCO_2 × plant composition	-0.203	0.408	0.622
	Aphid infestation	-0.128	0.286	0.659
	TCO_2 × plant composition × aphid infestation	0.186	0.574	0.748
	CO_2 × plant composition	-0.113	0.408	0.784
	Plant composition	0.060	0.291	0.838
	TCO_2 × aphid infestation	-0.064	0.404	0.876
	Plant composition × aphid infestation	-0.021	0.408	0.960

Table S2 Partial slopes of the structural equation model presented in Figure 2B. Labels CO_2 and TCO_2 indicate elevated CO_2 and combined warming and elevated CO_2, respectively. Plant communities consist of monocultures of *Plantago lanceolata* and mixtures of *P. lanceolata* and *Lolium perenne*. The four clusters refer to those obtained by the hierarchical clustering analysis (see Fig. 1). P values are presented in bold when significant (≤0.05).

Response	Predictor	Estimate	SE	P-value
Aphid population	cluster 2 controle	1.558	0.664	0.066
	CO_2	1.650	1.462	0.288
	TCO_2	1.149	1.088	0.319
	cluster 1 controle	-0.837	0.990	0.436
	Plant composition	-0.463	0.581	0.461
	cluster 3 controle	-0.218	0.310	0.512
	TCO_2 × plant composition	0.210	0.653	0.761
	CO_2 × plant composition	-0.315	1.043	0.774
	cluster 4 controle	0.185	1.140	0.878
Cluster 1 controle	CO_2	1.024	0.150	**0.000**
	Plant composition	0.502	0.131	**0.004**
	CO_2 × plant composition	-0.595	0.185	**0.011**
	TCO_2	0.456	0.150	**0.014**
	TCO_2 × plant composition	-0.154	0.185	0.426

Response	Predictor	Estimate	SE	P-value
Cluster 2 controle	CO_2	-0.591	0.235	**0.033**
	CO_2 × plant composition	0.247	0.241	0.333
	Plant composition	0.140	0.171	0.431
	TCO_2	-0.156	0.235	0.525
	TCO_2 × plant composition	-0.089	0.241	0.722
Cluster 3 controle	Plant composition	-0.599	0.339	0.111
	CO_2	-0.646	0.524	0.248
	TCO_2 × plant composition	-0.296	0.479	0.552
	CO_2 × plant composition	-0.259	0.479	0.602
	TCO_2	0.250	0.524	0.645
Cluster 4 controle	TCO_2	0.494	0.115	**0.002**
	CO_2	0.401	0.115	**0.007**
	CO_2 × plant composition	-0.372	0.148	**0.033**
	TCO_2 × plant composition	-0.243	0.148	0.136
	Plant composition	0.027	0.105	0.806

Response	Predictor	Estimate	SE	P-value
Cluster 1 aphids	Plant composition	0.518	0.327	0.212
	TCO_2 × aphid population × plant composition	-0.675	0.471	0.247
	CO_2 × plant composition	-0.526	0.402	0.281
	CO_2 × aphid population × plant composition	-0.536	0.462	0.329
	CO_2 × aphid population	-0.269	0.281	0.409
	Aphid population × plant composition	0.329	0.398	0.469
	Aphid population	0.133	0.200	0.555
	CO_2	0.115	0.257	0.664
	TCO_2	0.050	0.268	0.856
	TCO_2 × aphid population	0.017	0.255	0.952
	TCO_2 × plant composition	0.002	0.410	0.997
Cluster 2 aphids	CO_2	-1.525	0.200	**0.000**
	TCO_2	-1.590	0.208	**0.000**
	TCO_2 × aphid population × plant composition	-0.892	0.394	0.108
	CO_2 × aphid population × plant composition	-0.780	0.388	0.138
	TCO_2 × aphid population	0.375	0.199	0.155
	TCO_2 × plant composition	0.564	0.341	0.197

Response	Predictor	Estimate	SE	P-value
	Plant composition	0.431	0.269	0.207
	Aphid population × plant composition	0.531	0.333	0.209
	Aphid population	-0.227	0.155	0.241
	CO_2 × aphid population	0.266	0.218	0.310
	CO_2 × plant composition	0.278	0.334	0.466
Cluster 3 aphids	CO_2 × aphid population × plant composition	0.993	0.489	0.135
	Aphid population × plant composition	-0.857	0.440	0.147
	TCO_2 × aphid population × plant composition	0.675	0.500	0.270
	TCO_2	0.508	0.470	0.309
	CO_2	-0.492	0.460	0.312
	Plant composition	-0.501	0.429	0.327
	TCO_2 × aphid population	-0.400	0.402	0.392
	CO_2 × aphid population	-0.433	0.481	0.434
	Aphid population	0.264	0.344	0.498
	CO_2 × plant composition	0.363	0.476	0.501
	TCO_2 × plant composition	-0.156	0.483	0.768

Response	Predictor	Estimate	SE	P-value
Cluster 4 aphids	TCO_2	1.432	0.207	**0.000**
	CO_2	1.015	0.199	**0.001**
	TCO_2 × plant composition	-0.954	0.339	0.067
	CO_2 × plant composition	-0.618	0.333	0.160
	CO_2 × aphid population	-0.337	0.217	0.219
	Plant composition	-0.377	0.267	0.253
	Aphid population	0.214	0.155	0.260
	TCO_2 × aphid population	-0.150	0.198	0.504
	Aphid population × plant composition	-0.191	0.331	0.605
	CO_2 × aphid population × plant composition	0.218	0.386	0.611
	TCO_2 × aphid population × plant composition	0.096	0.391	0.823

5.7.3 Section 3: supplementary tables: results from GLM analyses

Table S1 Summary of GLM results for effects of climate scenario, plant composition and aphid infestation on the carbohydrate fractions in *Plantago lanceolata*. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*. P-values are presented in bold when significant (≤ 0.05).

Measurement	Treatment	df	F	P
Soluble sugars	Climate scenario	2,84	7.57	**0.001**
	Plant composition	1,84	4.97	**0.028**
	Aphid infestation	1,84	19.17	**<0.001**
	Climate scenario × aphid infestation	2,84	1.69	0.190
	Climate scenario × plant composition	2,84	10.68	**<0.001**
	Plant composition × aphid infestation	1,84	1.59	0.210
	Climate scenario × aphid infestation × plant composition	2,84	0.02	0.985
Starch	Climate scenario	2,84	23.51	**<0.001**
	Plant composition	1,84	75.83	**<0.001**
	Aphid infestation	1,84	35.69	**<0.001**
	Climate scenario × aphid infestation	2,84	2.29	0.108
	Climate scenario × plant composition	2,84	1.08	0.344
	Plant composition × aphid infestation	1,84	0.62	0.435
	Climate scenario × aphid infestation × plant composition	2,84	0.04	0.964

Measurement	Treatment	df	F	P
Sucrose	Climate scenario	2,84	9.24	**<0.001**
	Plant composition	1,84	21.14	**<0.001**
	Aphid infestation	1,84	30.22	**<0.001**
	Climate scenario × aphid infestation	2,84	8.07	**0.001**
	Climate scenario × plant composition	2,84	8.21	**0.001**
	Plant composition × aphid infestation	1,84	23.89	**<0.001**
	Climate scenario × aphid infestation × plant composition	2,84	2.52	0.0863
Glucose	Climate scenario	2,84	4.20	**0.018**
	Plant composition	1,84	0.63	0.428
	Aphid infestation	1,84	8.01	**0.006**
	Climate scenario × aphid infestation	2,84	6.77	**0.002**
	Climate scenario × plant composition	2,84	3.81	**0.013**
	Plant composition × aphid infestation	1,84	0.24	0.625
	Climate scenario × aphid infestation × plant composition	2,84	2.05	0.136

Measurement	Treatment	df	F	P
Fructose	Climate scenario	2,84	8.10	**0.001**
	Plant composition	1,84	0.4	0.529
	Aphid infestation	1,84	17.66	<**0.001**
	Climate scenario × aphid infestation	2,84	4.88	**0.001**
	Climate scenario × plant composition	2,84	1.14	0.323
	Plant composition × aphid infestation	1,84	0.12	0.732
	Climate scenario × aphid infestation × plant composition	2,84	0.08	0.928

Table S2 Summary of GLM results for effects of climate scenario, plant composition and aphid infestation on the defence molecules in *Plantago lanceolata*. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*. P-values are presented in bold when significant (≤ 0.05).

Measurement	Treatment	df	F	P
Lignin	Climate scenario	2,9	16.45	**0.001**
	Plant composition	1,75	67.05	**<0.001**
	Aphid infestation	1,75	81.49	**<0.001**
	Climate scenario × aphid infestation	2,75	14.64	**<0.001**
	Climate scenario × plant composition	2,75	8.24	**0.001**
	Plant composition × aphid infestation	1,75	8.27	**0.005**
	Climate scenario × aphid infestation × plant composition	2,75	3.14	**0.049**
Tannin	Climate scenario	2,84	23.51	**<0.001**
	Plant composition	1,84	0.22	0.638
	Aphid infestation	1,84	15.55	**<0.001**
	Climate scenario × aphid infestation	2,84	2.07	0.132
	Climate scenario × plant composition	2,84	8.61	**<0.001**
	Plant composition × aphid infestation	1,84	9.28	**0.003**
	Climate scenario × aphid infestation × plant composition	2,84	0.06	0.941

Measurement	Treatment	df	F	P
Cellulose	Climate scenario	2,84	0.14	0.870
	Plant composition	1,84	6.99	**0.010**
	Aphid infestation	1,84	8.66	**0.004**
	Climate scenario × aphid infestation	2,84	0.90	0.409
	Climate scenario × plant composition	2,84	0.14	0.866
	Plant composition × aphid infestation	1,84	12.04	**0.001**
	Climate scenario × aphid infestation × plant composition	2,84	4.05	**0.021**
Catalpol	Climate scenario	2,84	1.38	0.257
	Plant composition	1,84	6.73	**0.011**
	Aphid infestation	1,84	152.81	**<0.001**
	Climate scenario × aphid infestation	2,84	0.99	0.375
	Climate scenario × plant composition	2,84	0.74	0.480
	Plant composition × aphid infestation	1,84	5.92	**0.017**
	Climate scenario × aphid infestation × plant composition	2,84	0.20	0.821

Measurement	Treatment	df	F	P
Aucubin	Climate scenario	2,84	5.53	**0.005**
	Plant composition	1,84	1.31	0.255
	Aphid infestation	1,84	128.12	**<0.001**
	Climate scenario × aphid infestation	2,84	5.47	**0.006**
	Climate scenario × plant composition	2,84	1.29	0.280
	Plant composition × aphid infestation	1,84	1.31	0.255
	Climate scenario × aphid infestation × plant composition	2,84	1.04	0.357

Table S3 Summary of GLM results for effects of climate scenario, plant composition and aphid infestation on the macronutrients in *Plantago lanceolata*. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*. P-values are presented in bold when significant (≤ 0.05).

Measurement	Treatment	df	F	P
Leaf nitrogen	Climate scenario	2,9	2.89	0.108
	Plant composition	1,75	9.82	**0.003**
	Aphid infestation	1,75	4.74	**0.033**
	Climate scenario × aphid infestation	2,75	0.25	0.780
	Climate scenario × plant composition	2,75	0.49	0.615
	Plant composition × aphid infestation	1,75	1.95	0.167
	Climate scenario × aphid infestation × plant composition	2,75	0.15	0.857
Leaf carbon	Climate scenario	2,84	3.00	0.055
	Plant composition	1,84	8.00	**0.006**
	Aphid infestation	1,84	0.00	0.945
	Climate scenario × aphid infestation	2,84	1.65	0.199
	Climate scenario × plant composition	2,84	0.16	0.852
	Plant composition × aphid infestation	1,84	1.37	0.245
	Climate scenario × aphid infestation × plant composition	2,84	0.41	0.667

Measurement	Treatment	df	F	P
Phosphorous	Climate scenario	2,84	14.30	**<0.001**
	Plant composition	1,84	4.87	**0.030**
	Aphid infestation	1,84	5.53	**0.021**
	Climate scenario × aphid infestation	2,84	0.34	0.716
	Climate scenario × plant composition	2,84	2.21	0.116
	Plant composition × aphid infestation	1.84	5.00	**0.028**
	Climate scenario × aphid infestation × plant composition	2,84	0.71	0.495
Lipids	Climate scenario	2.84	29.66	**<0.001**
	Plant composition	1,84	13.88	**<0.001**
	Aphid infestation	1.84	61.04	**<0.001**
	Climate scenario × aphid infestation	2.84	5.93	**0.004**
	Climate scenario × plant composition	2,84	0.86	0.426
	Plant composition × aphid infestation	1,84	1.21	0.274
	Climate scenario × aphid infestation × plant composition	2,84	7.38	**0.001**

Measurement	Treatment	df	F	P
Total proteins	Climate scenario	2,84	23.41	**<0.001**
	Plant composition	1,84	1.42	0.238
	Aphid infestation	1,84	30.03	**<0.001**
	Climate scenario × aphid infestation	2,84	1.36	0.262
	Climate scenario × plant composition	2,84	2.81	0.066
	Plant composition × aphid infestation	1,84	0.01	0.924
	Climate scenario × aphid infestation × plant composition	2,84	2.46	**0.025**

Table S4 Summary of GLM results for effects of climate scenario, plant composition and aphid infestation on the stoichiometirc ratios in *Plantago lanceolata*. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*. P-values are presented in bold when significant (≤ 0.05).

Measurement	Treatment	df	F	P
C:N ratio	Climate scenario	2.84	8.71	**<0.001**
	Plant composition	1,84	6.74	**0.011**
	Aphid infestation	1,84	2.43	0.123
	Climate scenario × aphid infestation	2,84	0.38	0.684
	Climate scenario × plant composition	2,84	0.31	0.733
	Plant composition × aphid infestation	1,84	1.12	0.293
	Climate scenario × aphid infestation × plant composition	2,84	0.17	0.842
C:P ratio	Climate scenario	2,84	15.98	**<0.001**
	Plant composition	1,84	10.04	**0.002**
	Aphid infestation	1,84	8.66	**0.004**
	Climate scenario × aphid infestation	2,84	0.80	0.452
	Climate scenario × plant composition	2,84	3.66	**0.030**
	Plant composition × aphid infestation	1,84	5.61	**0.020**
	Climate scenario × aphid infestation × plant composition	2,84	0.35	0.703

Measurement	Treatment	df	F	P
N:P ratio	Climate scenario	2,9	7.81	**0.011**
	Plant composition	1,75	23.32	**<0.001**
	Aphid infestation	1,75	4.39	**0.040**
	Climate scenario × aphid infestation	2,75	1.50	0.230
	Climate scenario × plant composition	2,75	7.60	**0.001**
	Plant composition × aphid infestation	1,75	3.82	0.054
	Climate scenario × aphid infestation × plant composition	2,75	0.20	0.820
Lignin:N	Climate scenario	2,9	24.88	**<0.001**
	Plant composition	1,75	22.36	**<0.001**
	Aphid infestation	1,75	63.02	**<0.001**
	Climate scenario × aphid infestation	2,75	2.26	0.112
	Climate scenario × plant composition	2,75	3.43	**0.037**
	Plant composition × aphid infestation	1,75	5.18	**0.026**
	Climate scenario × aphid infestation × plant composition	2,75	1.07	0.348

Table S5 Summary of GLM results for effects of climate scenario, plant composition and aphid infestation on the oxidative stress indicator and the antioxidants in *Plantago lanceolata*. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*. P-values are presented in bold when significant (≤ 0.05).

Measurement	Treatment	df	F	P
Total antioxidant capacity	Climate scenario	2,84	5.97	**0.004**
	Plant composition	1,84	0.80	0.374
	Aphid infestation	1,84	261.74	**<0.001**
	Climate scenario × aphid infestation	2,84	0.12	0.889
	Climate scenario × plant composition	2,84	4.00	**0.022**
	Plant composition × aphid infestation	1,84	7.31	**0.008**
	Climate scenario × aphid infestation × plant composition	2,84	0.15	0.865
Total phenols	Climate scenario	2,84	12.68	**<0.001**
	Plant composition	1,84	23.24	**<0.001**
	Aphid infestation	1,84	79.59	**<0.001**
	Climate scenario × aphid infestation	2,84	18.15	**<0.001**
	Climate scenario × plant composition	2,84	1.41	0.250
	Plant composition × aphid infestation	1,84	2.40	0.125
	Climate scenario × aphid infestation × plant composition	2,84	0.17	0.845

Measurement	Treatment	df	F	P
Carotenoids	Climate scenario	2,84	9.17	**<0.001**
	Plant composition	1,84	14.48	**<0.001**
	Aphid infestation	1,84	5.67	**0.019**
	Climate scenario × aphid infestation	2,84	15.28	**<0.001**
	Climate scenario × plant composition	2,84	1.06	0.351
	Plant composition × aphid infestation	1,84	1.30	0.257
	Climate scenario × aphid infestation × plant composition	2,84	2.85	0.064
Tocopherols	Climate scenario	2,84	15.84	**<0.001**
	Plant composition	1,84	18.30	**<0.001**
	Aphid infestation	1,84	196.73	**<0.001**
	Climate scenario × aphid infestation	2,84	22.72	**<0.001**
	Climate scenario × plant composition	2,84	3.56	**0.003**
	Plant composition × aphid infestation	1,84	6.70	**0.011**
	Climate scenario × aphid infestation × plant composition	2,84	3.89	**0.024**

Measurement	Treatment	df	F	P
Proline	Climate scenario	2,84	29.37	**<0.001**
	Plant composition	1,84	22.55	**<0.001**
	Aphid infestation	1,84	138.62	**<0.001**
	Climate scenario × aphid infestation	2,84	24.19	**<0.001**
	Climate scenario × plant composition	2,84	11.63	**<0.001**
	Plant composition × aphid infestation	1,84	18.05	**<0.001**
	Climate scenario × aphid infestation × plant composition	2,84	4.78	**0.011**
Leaf membrane damage, MDA	Climate scenario	2,84	19.04	**<0.001**
	Plant composition	1,84	34.17	**<0.001**
	Aphid infestation	1,84	31.02	**<0.001**
	Climate scenario × aphid infestation	2,84	0.07	0.935
	Climate scenario × plant composition	2,84	2.65	0.077
	Plant composition × aphid infestation	1,84	3.84	0.054
	Climate scenario × aphid infestation × plant composition	2,84	2.31	0.105

Table S6 Summary of GLM results for effects of climate scenario, plant composition and aphid infestation on the hormones in *Plantago lanceolata*. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*. P-values are presented in bold when significant (≤ 0.05).

Measurement	Treatment	df	F	P
Jasmonic acid	Climate scenario	2,84	24.74	**<0.001**
	Plant composition	1,84	8.62	**0.004**
	Aphid infestation	1,84	2.06	0.154
	Climate scenario × aphid infestation	2,84	37.38	**<0.001**
	Climate scenario × plant composition	2,84	2.18	0.120
	Plant composition × aphid infestation	1,84	6.08	**0.016**
	Climate scenario × aphid infestation × plant composition	2,84	3.53	**0.034**
Salicylic acid	Climate scenario	2,84	15.71	**<0.001**
	Plant composition	1,84	150.51	**<0.001**
	Aphid infestation	1,84	390.48	**<0.001**
	Climate scenario × aphid infestation	2,84	21.69	**<0.001**
	Climate scenario × plant composition	2,84	6.70	**0.002**
	Plant composition × aphid infestation	1,84	110.21	**<0.001**
	Climate scenario × aphid infestation × plant composition	2,84	38.79	**<0.001**

5.7.4 Section 4: supplementary figures presenting results from GLM analyses

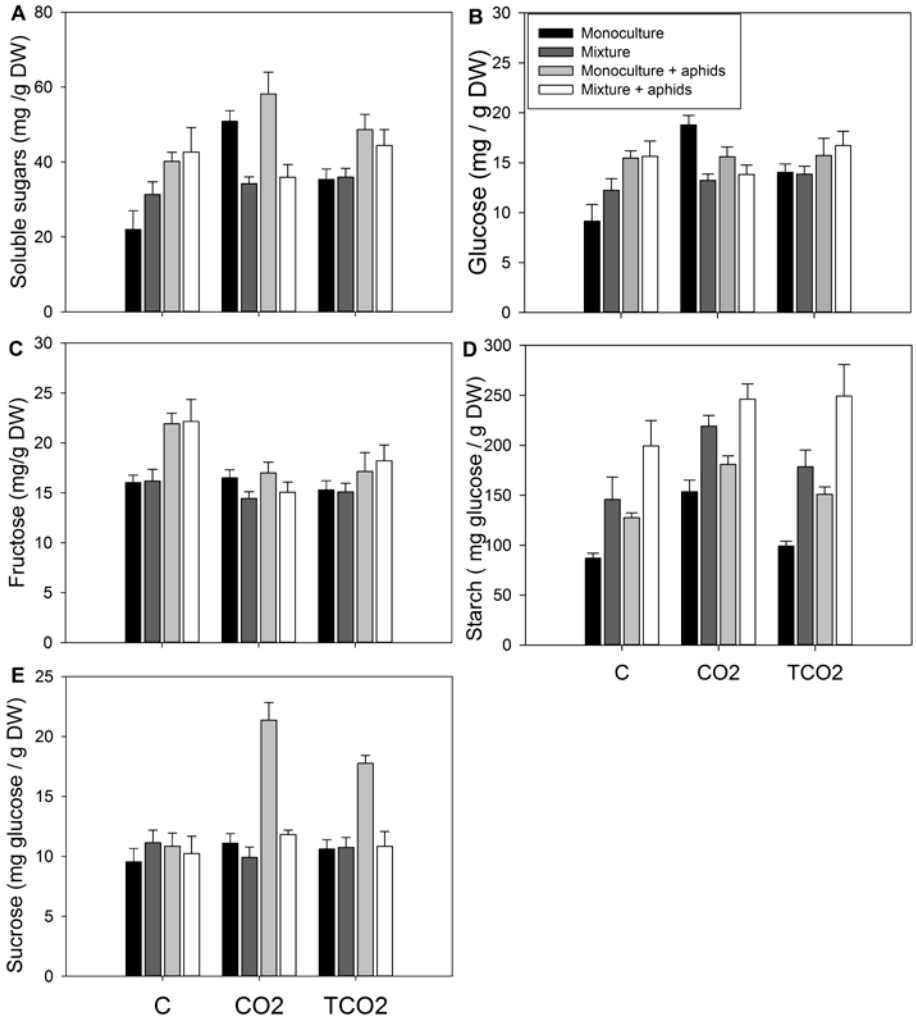

Fig. S1 Effect of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition on the carbohydrate fraction in *Plantago lanceolata*. Bars represent means ± SE. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*.

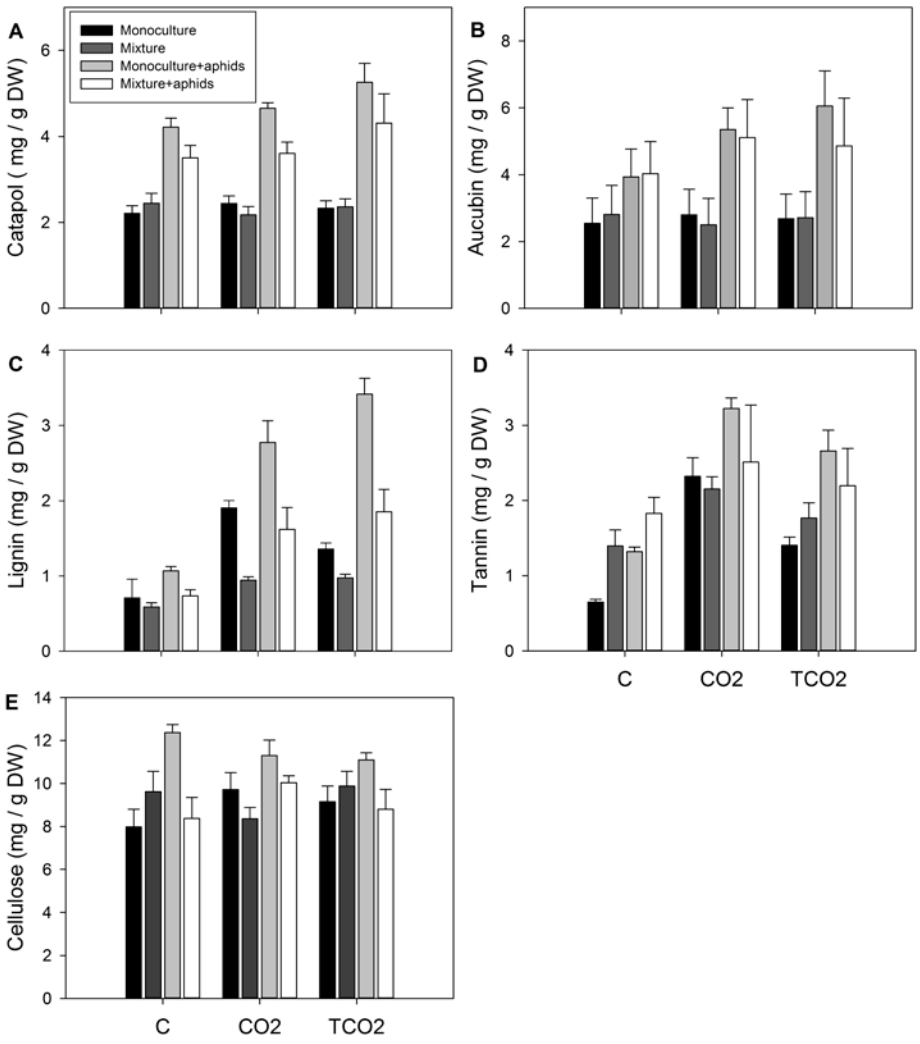

Fig. S2 Effect of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition on the defence molecules in *Plantago lanceolata*. Bars represent means ± SE. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*.

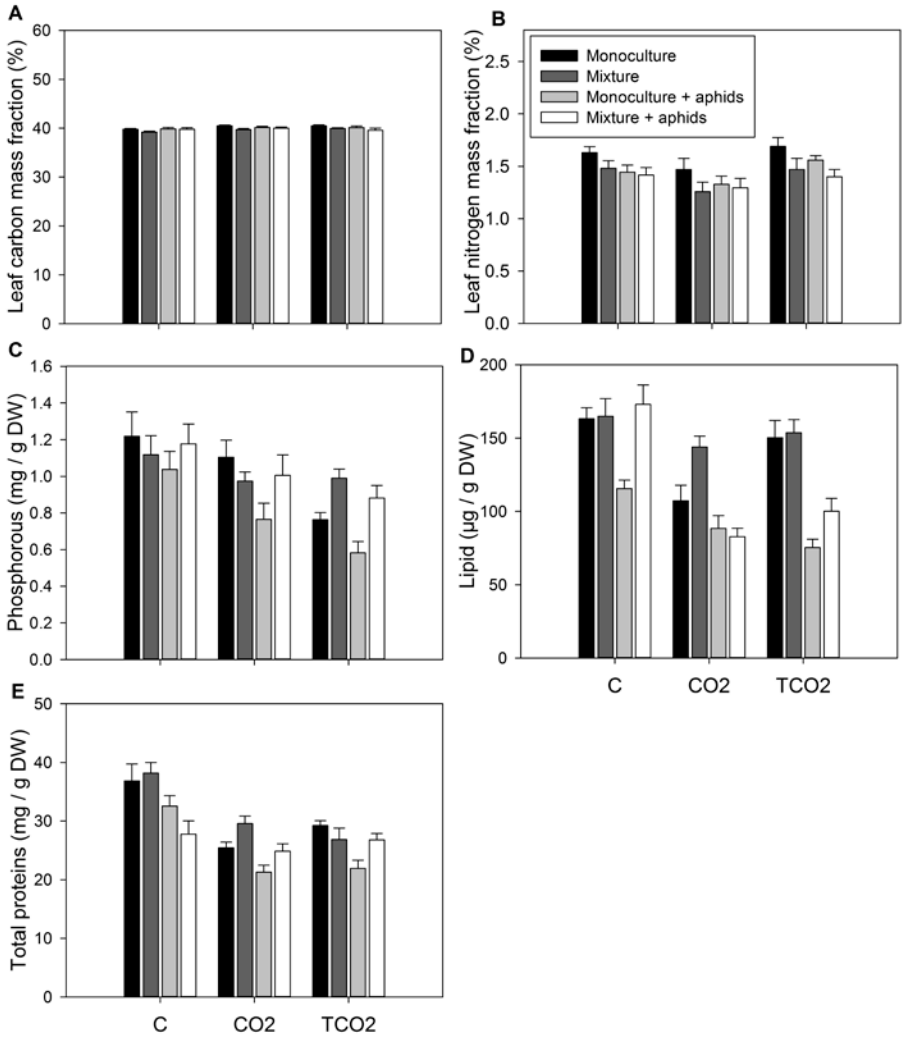

Fig. S3 Effect of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition on the macronutrients in *Plantago lanceolata.* Bars represent means ± SE. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata.*

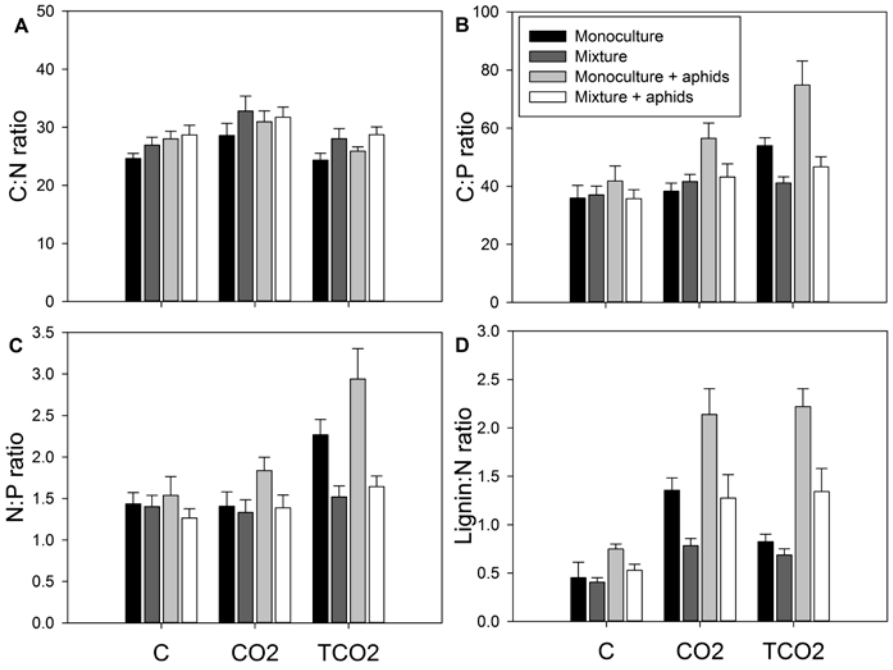

Fig. S4 Effect of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition on the stoichiometric ratios in *Plantago lanceolata.* Bars represent means ± SE. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*.

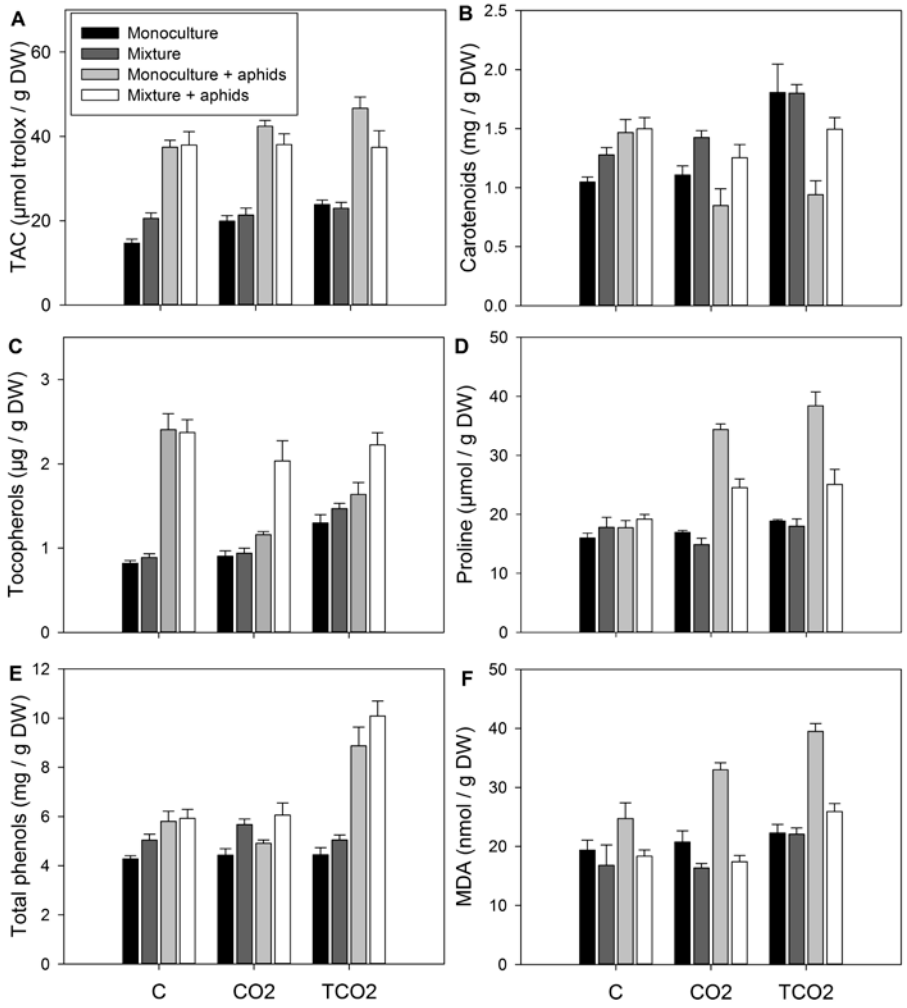

Fig. S5 Effect of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition on the oxidative stress indicator and the antioxidants in *Plantago lanceolata.* Bars represent means ± SE. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*.

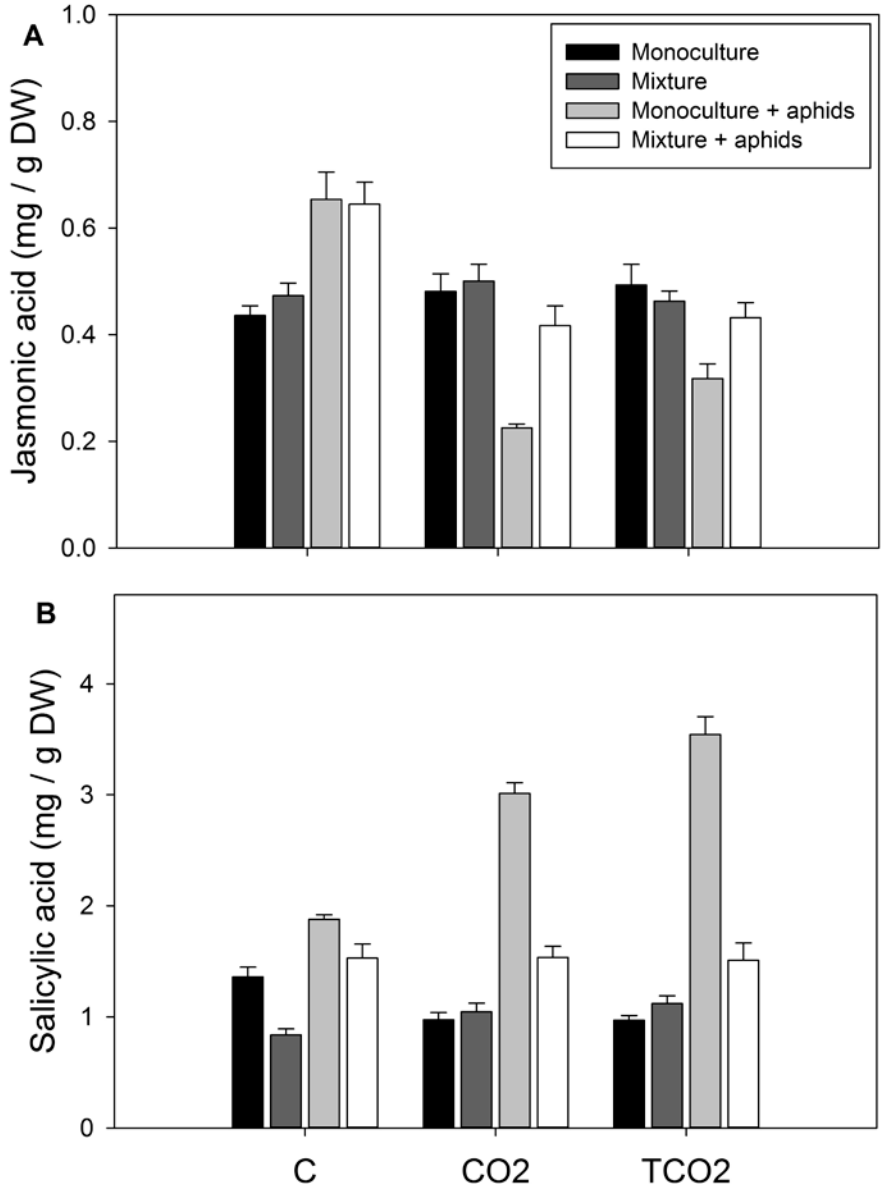

Fig. S6 Effect of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition on the hormones in *Plantago lanceolata*. Bars represent means ± SE. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*.

6 GENERAL DISCUSSION

Helena Van De Velde

6.1 OVERVIEW OF THE MAIN RESULTS

In the previous chapters we examined the independent and combined effects of elevated CO_2 and warming on stress responses of grassland species. In a first step, we explored the drought response and possible lagged effects of drought in a future climate. In a second step, we focused on the impact of a future climate on grassland species exposed to aphid herbivory. We analysed whether a future climate altered the plant-herbivore interaction. In addition to single species responses to a future climate, we particularly aimed to determine whether a future climate indirectly influenced the stress response of grassland species via effect on neighbouring plants. In addition to these experimental studies with drought and herbivory stress, we used an energy balance model and field data to examine the leaf temperature under various environmental conditions and the potential for heat stress. We obtained the following main results:

Using an energy balance model and field data we demonstrated that at the same air temperature, specific atmospheric conditions such as relative humidity, wind speed and radiation critically affect leaf temperatures, depending on plant water status. The interaction between a plant and its environment can cause leaf temperature fluctuations of 10 °C (for narrow leaves) to even 20 °C (for big broad leaves) at the same air temperature. We clearly demonstrated that heat stress depends on more variables than air temperature alone. Therefore, heat waves characterized by extreme air temperatures may pose little plant danger under some atmospheric conditions, while less high air temperatures may be lethal to plants in other cases. Our results can help to predict when the probability of heat stress is most likely.

Warming aggravated the drought impact on *Lolium perenne* by reducing PSII photochemical efficiency and increasing leaf mortality and senescence. Elevated CO_2 seems to alleviate the stress impact caused by warming and drought through increased photochemical protection but not by decreasing the necromass. Neither warming nor elevated CO_2 did modify the live biomass response of *L. perenne* and *Plantago lanceolata* to drought, suggesting that the plants were equally restrained by water shortage under current and future climate conditions. Contrary to *L. perenne*, warming or elevated CO_2 did not alter the drought response of *P. lanceolata*.

Remarkably, combined warming and drought, with or without elevated CO_2 induced higher senescence and mortality of *L. perenne* long after the drought ended, while no such lagged effects were apparent in the current climate. *P. lanceolata* also exhibited post-drought lagged effects on senescence and mortality, but only under combined warming and elevated CO_2. In general, plant composition did not alter the individual responses of *L. perenne* and *P. lanceolata* to drought in a future climate with warming and elevated CO_2.

Warming did not alter the live aboveground biomass and the PSII photochemical efficiency of *P. lanceolata* when exposed to aphid herbivory. In addition, we did not find indications for indirect effects of warming on *Dysaphis plantaginea* through changes in leaf quality. Despite this, warming affected the life history traits of the aphid directly, in a non-linear manner. The aphid performed best at moderate warming, where they were larger, had a shorter generation time and grew faster. Also plant competition affected aphid performance but through an interaction with temperature. Although warming affected aphid performance, the relative biomass losses of *P. lanceolata* did not alter under warming and consequently the herbivory rates were not changed. We demonstrated that when taking plant-plant interactions into account, the net interactions between plants and herbivores should not change under warming despite direct effects of warming on herbivores.

Aphid herbivory decreased essential nutrients, induced chemical defence molecules but did not alter the live aboveground biomass of *P. lanceolata*. *P. lanceolata* was protected against oxidative stress due to aphid herbivory by increased levels of antioxidants. Altogether, *P. lanceolata* showed induced direct resistance against the aphid *P. plantaginea*. The combined effect of elevated CO_2 and warming increased the induction of oxidative stress due to aphid herbivory but the different antioxidant molecules showed an opposite response. Elevated CO_2 is found to reduce the leaf quality of *P. lanceolata*. Also, simultaneously increasing both CO_2 and temperature affected the leaf quality but differently for different stoichiometric components. Furthermore, elevated CO_2, with or without warming enhanced both the systemic and the induced defence system. Surprisingly, interspecific plant competition neutralized the effect of elevated CO_2 on the defence molecules of *P. lanceolata*. Notwithstanding the significant effects of a future climate scenario on leaf quality and defence molecules, we did not find evidence for

indirect effects on aphid performance. In addition, we did not observe any direct effects of warming and elevated CO_2 on aphid performance. We demonstrated that multispecies responses can mediate single species responses to climate change.

The above-mentioned results will be discussed and evaluated in the last chapter. As the overall goal of climate change experiments is, building reliable forecasts of climate change effects, the discussion of the results has been conceived accordingly. In conclusion, directions for future research will be recommended.

6.2 STRESS SENSITIVITY OF *LOLIUM PERENNE* AND *PLANTAGO LANCEOLATA* IN A FUTURE CLIMATE

We studied the independent and combined effects of experimental warming and elevated CO_2 on the drought response and herbivory response of two grassland species *Lolium perenne* and *Plantago lanceolata*. In **chapter III and V**, we have discussed the individual effects of warming and elevated CO_2 on plant stress responses. Here, I focus on the comparison between stress in the current climate (with current temperature and CO_2) and stress in the future climate with simultaneously warming and elevated CO_2, as this comparison tells us how the stress response of grassland species will most probably alter in the future.

6.2.1 Metabolic modifications

An essential part of a plant's stress response (both biotic and abiotic), is the production of reactive oxygen species (ROS) (Mittler, 2002; Wu & Baldwin, 2010). To avoid the deleterious effects of ROS, plants have developed an antioxidant defence system (Mittler, 2002). Drought in a future climate reduced the concentrations of carotenoids of *L. perenne* to a similar level as the current climate without drought stress (**chapter III**). As an antioxidant, carotenoids protect the photosynthetic apparatus against photo-oxidative damage by scavenging reactive oxygen species (ROS) formed during photo-oxidative stress (DemmigAdams & Adams, 1996; Mittler, 2002; Telfer, 2005). Therefore, lower carotenoids concentrations under drought in a future climate may indicate a reduction in ROS formation. This may be ascribed to elevated CO_2 as the level of carotenoids in the climate scenario drought and warming was similar to drought in the current climate. Therefore, elevated CO_2 mitigated the oxidative stress of *L. perenne*. According to the relaxation hypothesis, reduced ROS formation in elevated CO_2 may result from decreased photorespiration (Foyer & Noctor, 2009; AbdElgawad *et al.*, 2016). Alternatively, the stress mitigation effect of elevated CO_2 can also be explained by the antioxidant hypothesis. According to this hypothesis, plants are better protected against oxidative damage in elevated CO_2 due to the increased supply of antioxidant molecules (Zinta *et al.*, 2014; AbdElgawad *et al.*, 2016). Higher antioxidant levels may result from increased internal availability of C under elevated CO_2. However, our study does not support

the antioxidant hypothesis as the antioxidant molecule carotenoid decreased in a future climate. Even with lower carotenoids concentrations, the production of ROS may still high in drought in a future climate. Lower levels of carotenoids to scavenge ROS means oxidative stress. However, this would be in contrast to previous research that found that elevated CO_2 mitigated the abiotic stress impact at the level of stress-generated reactive oxygen species (Geissler *et al.*, 2010; Mishra *et al.*, 2013; Zinta *et al.*, 2014; AbdElgawad *et al.*, 2015). We cannot exclude it.

A growing body of evidence points to the involvement of ROS in plant defences against insect herbivores (Kerchev *et al.*, 2012). ROS may negatively affect the digestive system of insects through membrane damage (Smith & Boyko, 2007) but also plants themselves may be harmed by ROS. Therefore, plants must find a balance between producing ROS for defence and producing antioxidants to help stabilize plant tissue damage due to oxidative degradation (Thompson & Goggin, 2006). A future climate has the potential to alter this balance, as seen for abiotic stress impact. However, in **chapter V** we found that a future climate did not alter the induction of oxidative stress and the antioxidant defence system of *P. lanceolata*. In contrast, a future climate enhanced both induced and systemic defence as it increased defensive metabolites in *P. lanceolata* plants with and without aphids. This positive effect of a future climate can be ascribed mainly to elevated CO_2. Elevated CO_2 increased specific defence molecules aucubin, lignin and tannin but did not alter catapol and cellulose. The carbon-nutrient-balance hypothesis predicts that these defence molecules should increase under elevated CO_2 as result of the 'excess' carbon (Bryant *et al.*, 1983). However, our study together with other studies, showed that the carbon-nutrient-balance hypothesis fails as predictive hypothesis (Hamilton *et al.*, 2001; Lindroth, 2012). New studies have proposed that resource utilisation to chemical defence is connected with photosynthesis, hormone regulation and control of gene expression (Zavala *et al.*, 2017). Besides mitigation of the abiotic stress impact, elevated CO_2 may also mitigate the biotic stress impact by enhancing the induced defence system.

6.2.2 Biomass response as measure of stress sensitivity

A future climate with elevated CO_2 and warming may alter a plant's growth response to stress as a consequence of an altered plant defence system. It has been shown that a future climate mitigates stress effects of drought, zinc toxicity, nitrogen limitation and heat at the level of the aboveground biomass production, i.e. the biomass production was reduced less in future than in current climate (Van den Berge *et al.*, 2011; Bauweraerts *et al.*, 2013; Naudts *et al.*, 2014; Zinta *et al.*, 2014; AbdElgawad *et al.*, 2015). To compare our results with the extant literature, I have made a plot of the biomass productivity of *L. perenne* and *P. lanceolata* in current and future climate conditions prior to the drought period (Fig. 1A) and after the drought period (Fig. 1C). The biomass productivity of both species in current and future climate conditions prior to and after the herbivory period can been found in Fig. 1B and Fig. 1D, respectively. Note that only *P. lanceolata* was exposed to aphid infestation.

Drought did not alter the biomass productivity of *P. lanceolata* neither in the current climate nor in the future climate (Fig. 1C). However, drought in a current climate reduced the biomass productivity of *L. perenne*. The biomass production of *L. perenne* in a future climate with drought tended to increase to an intermediary level, between the current climate and the current climate with drought (Fig. 1C). As the relative growth rate during drought did not differ between these two climate scenarios, this possible mitigation of the negative impact of drought in a future climate may be explained by a beneficial effect of the future climate before the drought, rather than through an effect of the future climate on drought. Indeed, combined warming and elevated CO_2 tended to increase the biomass production of *L. perenne* before the drought (Fig. 1A). In agreement with other studies, a future climate may not alter the impact of drought on the biomass productivity of *L. perenne* (Naudts *et al.*, 2011; Farfan-Vignolo & Asard, 2012; Naudts *et al.*, 2014). In other words, a future climate did not mitigate the stress impact at the level of biomass production. However, the future climate increased the dead biomass of *L. perenne*. This effect of a future climate can be ascribed mainly to warming as the climate scenario warming and drought did not differ from the climate scenario combined warming, elevated CO_2 and drought. Yet, the positive effect of elevated CO_2 was too weak to mitigate the biomass loss resulting from drought and warming.

In **chapter III** we found that a future climate did not alleviate the stress impact on biomass productivity. Nevertheless, the future climate induced lagged effects of drought by increasing the fraction of dead biomass in *L. perenne* and *P. lanceolata*. This higher fraction of dead biomass can be attributed mainly to warming as elevated CO_2 did not compensate for negative warming effects. The observed lagged effect on the fraction of dead biomass was not due to a difference in soil moisture. The mechanism behind the observed lagged effects could not be found within the measured parameters (see chapter III for more details). Moreover, we cannot completely exclude warming itself as a mechanism for lagged effects in *L. perenne* as just after the drought the dead fraction in the climate scenario drought and warming was significantly higher than in drought in a current climate. Studies have shown that warming can induce lagged effects on spring and autumn biomass production, soil respiration or flowering phenology (Sherry *et al.*, 2008; Zhou *et al.*, 2010; Sherry *et al.*, 2011). In contrast to our study, the results from these studies indicate that a lag in water recharge after experimental warming regulates the lag effects of warming on biomass production and flowering phenology. In addition, the higher fractions in dead biomass in the warming treatments at the end of the season in our study are not real lagged effects because the experimental warming is still going on. It is possible that it takes time to see the effect of warming.

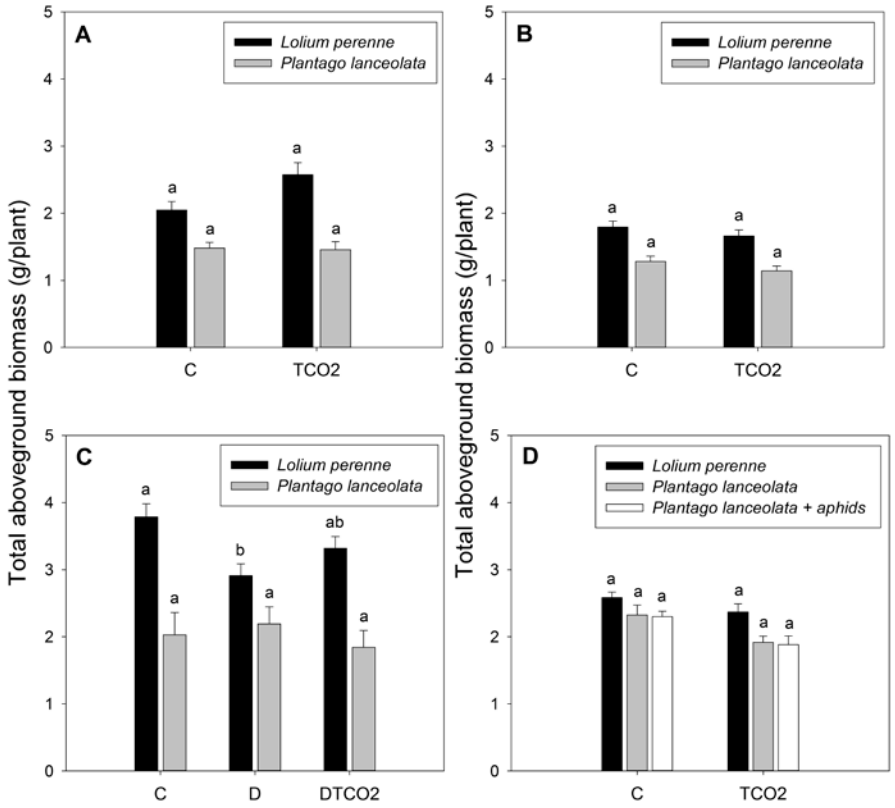

Fig. 1 Total aboveground biomass of *Lolium perenne* L. (black) and *Plantago lanceolata* L. (grey) before the drought (A) and the herbivory (B) period and after the drought (C) and the herbivory (D) period. White bars represent *P. lanceolata* infested with aphids. Plants were grown in current climate conditions (C), current climate with drought (D), warmer climate with elevated CO_2 (TCO_2) and warmer climate with elevated CO_2 and drought ($DTCO_2$). Means ± SE are indicated (all community compositions combined). Letters indicate differences for posterior comparisons between climate treatments, separately tested for each plant species.

In contrast to our laboratory experiment (**chapter IV**), aphid herbivory did not alter the biomass production of *P. lanceolata* under semi-natural conditions (Fig. 1D). It has been shown that *P. lanceolata* has the ability to compensate for biomass lost due to herbivory (Bowers & Stamp, 1993). Why were we not able to detect this under strict laboratory conditions? One of the reasons may be that in our laboratory experiment, plants were grown in growth chambers under artificial light. Due to low light conditions, carbon availability might have been too low to allow plants to compensate for lost

tissue. Surprisingly, combined warming and elevated CO_2 did not induce herbivory effects on the biomass production of *P. lanceolata* (Fig. 1D) and the relative biomass losses of *P. lanceolata*. In other words, herbivory rates were not altered under combined warming and elevated CO_2 (see paragraph: importance of species interactions).

In conclusion, a future climate with elevated CO_2 and warming mitigated abiotic and biotic stress impacts of grassland species at the level of a plant defence's system but not at the level of biomass production. The mitigation effects of a future climate on plant stress responses may be attributed to elevated CO_2.

6.3 TOWARDS MORE RELIABLE FORECAST OF GRASSLAND COMMUNITY RESPONSES UNDER CLIMATE CHANGE

There is an urgent need for more accurate predictions about species responses to climate change for a more effective protection of biodiversity. Most current models fail to make such accurate predictions as they ignore important biological mechanisms and instead use correlations between current species' occurrence and climatic and environmental variables (Urban, 2015). In a recent review, Urban *et al.* (2016) identify six biological mechanisms that are highly relevant for species responses to climate change but are too often missing from current predictive models: physiology, demography, dispersal, species interactions, evolution and environment (Fig. 2). They call for increased efforts to collect the data needed to parameterise biologically realistic predictive models in order to improve predictions of biological responses to climate change. In this PhD thesis we collected data to determine the role of three mechanisms, namely physiology, species interactions and demography in responses of grassland communities to climate change. The importance of these three mechanisms will be discussed in the next sections, based on the collected data.

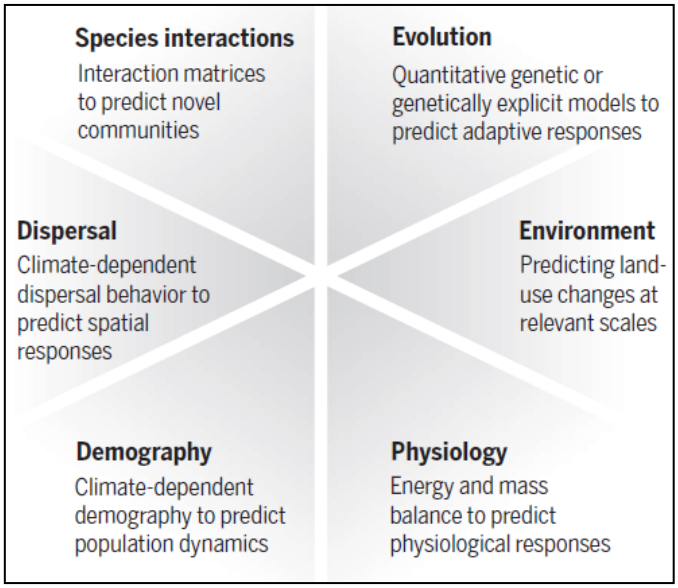

Fig. 2 Six key biological mechanisms that can improve predictions of biological responses to climate change (adapted from Urban *et al.*, 2016).

6.3.1 Leaf temperature as predictor of physiological responses to climate change

There is an urgent need for more information about physiological responses to extreme heat or drought (Urban *et al.*, 2016). To measure the impact of heat stress on plant responses, we must focus on leaf temperatures as plant physiological processes and metabolic rates are affected by leaf temperatures rather than air temperatures. In **chapter II**, we demonstrated that leaf temperatures can fluctuate considerably (10 °C for narrow leaves to even 20 °C for broad leaves) at constant air temperatures because other atmospheric conditions critically affect leaf temperatures, depending on plant water status. Therefore, as already suggested by other authors, it seems that many warming studies which only consider air temperatures may in some cases under- or overestimate the degree of warming and the impact of warming on plant metabolic processes (Scherrer & Koerner, 2010; De Boeck *et al.*, 2012).

Air temperatures are not only an inferior predictor for the impact of warming on plant metabolic processes but also for the impact of warming on folivorous insects (including aphids) that are in intimate contact with leaves (Pincebourde & Casas, 2006). Small increases in leaf temperatures can strongly stimulate the metabolism and consumption rates of insects (Zavala *et al.*, 2013). As elevated CO_2 decreases stomatal conductance, elevated CO_2 may indirectly increase leaf temperature. O'Neill *et al.* (2011) have already demonstrated that elevated CO_2 indirectly increased population growth of soybean aphids (*Aphis glycines* Matsumura) through increased leaf temperature. However, in **chapter II** we showed that apart from the stomatal response of the plant, a number of environmental conditions determine leaf temperatures. Therefore, differences in leaf temperatures caused by atmospheric conditions other than elevated CO_2, may explain some of the variation in response of insect herbivores to elevated CO_2.

In general, microclimatic conditions may mediate species responses to macroclimatic warming (Wallisdevries & Van Swaay, 2006; Scherrer & Koerner, 2010; De Frenne *et al.*, 2013; Maclean *et al.*, 2015). For instance, cooler forest-floor temperatures via increased shading during the growing season in denser forest, may buffer understory plant responses to macroclimate warming (De Frenne *et al.*, 2013). On the other hand,

microclimatic cooling in spring by advancing plant growth owing to global warming and nitrogen deposition may decline spring–developing thermophilous butterflies who rely on warm spring microclimates (Wallisdevries & Van Swaay, 2006).

It is clear that we cannot predict local or regional species losses based on rising air temperatures alone. Our study stresses the importance of measuring microclimatic conditions and particularly leaf temperature in order to accurately investigate the impact of climate warming and elevated CO_2 on plants species and plant species interactions.

6.3.2 Importance of demographic data and life history traits

The quantification of life history traits is fundamental to enable reliable forecasting of species responses to climate change (Selwood *et al.*, 2015; Urban *et al.*, 2016). In this thesis, we used life history traits to characterize aphid population dynamics.

6.3.2.1 Direct effects of warming on aphids

As confirmed in **chapter IV** and in several other studies, warming directly affects the life history traits of insect herbivores (Bale *et al.*, 2002; Zvereva & Kozlov, 2006; Van Baaren *et al.*, 2010; Jamieson *et al.*, 2012). Aphid performance increased with rising temperature (shorter generation times and stronger exponential growth) until the upper temperature threshold, which is 20 °C in the case of the aphid *Dysaphis plantaginea*. Increased rates of development are likely to lead to a higher number of generations per year if rising temperatures increase the length of the host-plant growing season (Bale *et al.*, 2002; Ziter *et al.*, 2012).

Most studies on the effect of warming on aphids or insects in general, have focused on the impact of development or reproduction, processes that occur predominantly in the summer. However, also the mean winter temperature will increase during the near future. In warm temperate climates many aphid species reproduce parthenogenetically the whole year by producing female clones. In cooler climates, aphids overwinter as cold-hardy eggs to avoid cold winter temperatures (Dixon, 1998). However, most pest aphid species display both types of reproduction. Within northern Europe it seems that

winter minimum temperature are not a threat to survival as the minimum temperature threshold for survival of eggs tends to be considerably lower than the minimum temperature of their winter environments (Bale *et al.*, 2007). However, the overwintering 'active stages' are not very tolerant to low temperatures. Therefore, mild winter increases the survival of those aphids in the active stages (Bale & Hayward, 2010). Both increased voltinism (number of generations in a year) and recued overwintering mortality is likely to have significant consequences for crop protection and production (Bale & Hayward, 2010; Barbosa *et al.*, 2012; Ziter *et al.*, 2012).

Above the temperature threshold, thermal stress and higher risk of mortality appears (Hazell *et al.*, 2010), which in our experiment lowered the maximum number of aphids and increased the generation time. Despite this, this level of warming still accelerated the exponential growth of the population by means of higher fecundity (Meisner *et al.*, 2014; Ramalho *et al.*, 2015). Because of thermal stress, experimental heat waves have been shown to decrease the fecundity, population growth and abundance of aphids (Gillespie *et al.*, 2012; Sentis *et al.*, 2013).

6.3.2.2 Plant-mediated effects of warming on aphids

Aphids use their flexible and long stylets to obtain nutrients from the phloem sap (Dixon, 1998). Phloem sap is rich in carbohydrates but insufficient in nitrogenous compounds (Mittler, 1953). Therefore, aphids consume large quantities of sugar-rich sap and excrete the majority of phloem sugars as honeydew in order to meet their minimum nitrogen requirements (Mattson, 1980). Free amino acids are the principal nitrogenous compounds in phloem sap (Douglas, 1993). Studies have shown that plant amino acid composition and concentration have the strongest effects on aphid performance compared with other metabolites (Douglas, 1993; Dixon, 1998; Karley *et al.*, 2002). Aphids cannot synthesise ten of the twenty essential amino acids (required for survival) (Douglas, 1993). The primary intracellular bacterial symbionts *Buchnear aphidicola* supply these essential amino acids, in exchange for other nutrients (Houk & Griffiths, 1980). Because of this exchange, aphids can utilise a nutritionally poor and imbalanced phloem diet which has generally a low essential amino acid content (Ryan *et al.*, 2015).

In **chapter IV and V,** we investigated indirect effects of warming on aphid performance. Note that in **chapter V**, we did not investigate the independent

effect of warming; however, the results are received by comparing the combined effect of warming and elevated CO_2 with elevated CO_2 alone. Warming decreased the concentrations of P, had little effect on leaf N concentrations and no effect on leaf proteins of *P. lanceolata*. However, the quality of plant tissue for insect herbivores depends not only on the concentration of essential nutrients but also on the concentration of defensive secondary compounds. We did not find an indication of effect of warming on defence molecules of *P. lanceolata*. Consequently, warming had minimal effects on leaf quality in both experiments, and thus we could not detect indirect effects of warming on aphid performance. However, in these experiments we tried to relate performance to whole-tissue chemistry while the plant-mediated mechanism underlying responses to climate change is likely driven by changes in plant amino acids compositions and concentrations in phloem (Sun & Ge, 2011; Ryan *et al.*, 2014; Ryan *et al.*, 2015; Ryalls *et al.*, 2017). Ryalls *et al.* (2017) showed that warming decreased the amino acid concentrations due to a decline in specific amino acids. As we did not measure amino acid concentrations in phloem, we cannot exclude indirect effects of warming on aphid performance in our experiments.

6.3.2.3 Combined effect of warming and elevated CO_2 on aphids

In our field experiment, we did not measure different life history traits of the aphid for practical reasons (**chapter V**). We used aphid abundance to investigate the impact of elevated CO_2 and combined warming and elevated CO_2 on aphid performance.

Predicting the response of aphids to elevated CO_2 is difficult. Several studies have detected an improvement in aphid performance under elevated CO_2 (Awmack *et al.*, 1997; Hughes & Bazzaz, 2001; Ryan *et al.*, 2015; Ryalls *et al.*, 2017), whereas others have noticed decreases (Awmack *et al.*, 2004; Ryan *et al.*, 2014) and still others have found no change (Diaz *et al.*, 1998; Hughes & Bazzaz, 2001). This has lead to the suggestion that aphid responses to elevated CO_2 are idiosyncratic (Pritchard *et al.*, 2007; Sun & Ge, 2011). However, using mechanism such as density dependence, temperature-dependent aphid development rates, aphid nitrogen requirement and nitrogen soil fertility, mathematical models were able to account for the

diversity of aphid responses to CO_2 (Newman *et al.*, 2003; Hoover & Newman, 2004; Newman, 2004; Newman, 2005). Therefore, it cannot be ruled out that aphid responses are controlled by a general mechanism.

We did not find significant effects of elevated CO_2 or combined warming and elevated CO_2 on aphid performance. In this experiment, we artificially defined the end of the aphid infestation (after three weeks) and thus the number of aphids may not match the threshold value for dispersal of aphids when plant conditions are sub-optimal, as in **chapter IV**. Therefore, the lack of a significant effect of future climate on aphid performance may be due to too short aphid infestation. Furthermore, abundances are 'static' rather than dynamic measures, and thus generally do not provide much information on trajectories of change and become unreliable over time (Selwood *et al.*, 2015; Urban *et al.*, 2016). Perhaps life history traits might have shed light on the effects of warming and elevated CO_2 on aphid performance in our field experiment.

Up till now, only a few studies have shown that elevated CO_2 may increase the relative concentration of essential amino acid in the phloem (Ryan *et al.*, 2015; Ryalls *et al.*, 2017). This result suggests that amino acid biosyntheses and translocation in some non-legumes and legumes may increase under elevated CO_2 although the total N concentrations of plants is decreased. This may explain observations of improved phloem feeder performance under elevated CO_2.

Elevated CO_2 has not only an effect on primary but also on secondary metabolism. Secondary metabolites may help the plant to resist aphid attack by negatively affecting the penetration pathway stage of aphids feeding. For example, aphids required a prolonged time to penetrate the epidermis and mesophyll when feeding on high-saponin lines of alfalfa, which in turn reduced the phloem sap ingestion (Goławska, 2007). As confirmed in chapter V, elevated CO_2 enhances salicylic-dependent defence which may lead to increased epidermis and mesophyll resistance of plants during probing feeding stages of aphids under elevated CO_2 (Guo *et al.*, 2014). However, elevated CO_2 decreased jasmonic acid pathway, which reduced the total time required by aphids to reach phloem (Sun *et al.*, 2016). As suggested in **chapter V** and demonstrated in previous research, despite increasing defence molecules aphids performed better under elevated CO_2 (Bezemer *et al.*, 1998; Zhang *et al.*, 2003). This result suggests that aphids

can avoid some potential defensive compound due to their tricky feeding strategy (Sun *et al.*, 2016). It is clear that the effects of elevated CO_2 on the interaction between plants and aphids cannot be understood by examining the effects of one aspect of plant quality to a specific feeding phase of the aphid.

Only a handful of empirical studies have examined the combined effect of warming and elevated CO_2 on aphids. For instance, Flynn *et al*. (2006) suggested that the potato aphid (*Macrosiphum euphorbiae*) will increase under combined warming and elevated CO_2 and thus exacerbate the damage to the C_3 perennial *Solanum dulcamara*. In legumes, however, aphid performances increased under elevated CO_2 but warming negated the positive effects of elevated CO_2 (Ryalls *et al.*, 2015; Ryalls *et al.*, 2017). These effects of a future climate was ascribed to altered amino acid concentrations. Warming counteracted any positive effects of elevated CO_2 on amino acid concentrations. Determining the concentrations of the right metabolites is essential to better understand the impact of a future climate on aphid performance.

Besides physiological and biochemical plant responses to climate change, detailed demographic data are also necessary to forecast climate change impact on plant populations. PlantPoPNet is a recently launched globally distributed Plant Population Dynamics Network, that studies the long-term demographic performance of *P. lanceolata* under contrasting environmental conditions and in interaction with other organisms. The researchers like to answer important questions about the environmental and biological drivers of population performance and extinction, how plants adjust their life history strategies in different environments, and what are the demographic mechanisms of plant invasion. Data sets collected by teams like PlantPopNet will enable more mechanistic modelling of species responses to climate change at continental or global scales.

6.3.3 Importance of species interactions

A majority of experimental studies and models focus on single species responses to climate change and assume that each species responds independently (Davis *et al.*, 1998; Gilman *et al.*, 2010). This assumption may hold in specific cases. For instance, in **chapter III**, we demonstrated that plant-plant interactions did not alter the individual response of *Lolium*

perenne and *Plantago lanceolata* to a combination of drought and warming or drought, warming and elevated CO_2. We expected that both species would compete significantly during drought because of similar rooting depth (Weeve, 1975) and a different capacity for water acquisition and transport and that species-specific productivity responses in a future climate with drought would alter competitive interactions. However, *L. perenne* can suppress the root production of herbaceous species, especially in top soil (Wardle & Peltzer, 2003). Limited interaction between the *L. perenne* and *P. plantago* owing to root partitioning may be the reason that we did not find altered plant-plant interactions under climate change and therefore also no indirect effects of climate change on both species.

In **chapter V** we demonstrated that plant-plant interactions modified the impact of climate change on leaf quality and the defence system of *P. lanceolata*. It is well-known that interaction between a focal plant and its insect herbivore can be strongly influenced by neighbouring plants. Neighbouring plants may affect the plant size, leaf quality and secondary chemicals of the focal plant (Fig. 3 pathway A) (Agrawal, 2004; Barton & Bowers, 2006; Broz *et al.*, 2010). The identity of a neighbouring plant plays an important role (Barton & Bowers, 2006; Broz *et al.*, 2010). Indeed, *L. perenne* as a neighbour mediated the induced response of some metabolites in *P. lanceolata* in the current climate and the induced and systemic response in a future climate compared when surrounded by conspecific neighbours. More specific, interspecific plant interactions mitigated the positive effect of elevated CO_2 on the induced response of the defence molecules lignin, catalpol and cellulose. As discussed in more detail in chapter V, the down-regulation of plant defences under the influence of competition is directly mediated by light signals (Ballaré, 2014; Campos *et al.*, 2016), most notably the ratio of red to far-red light (Ballaré *et al.*, 1990). This implies that *P. lanceolata* was more constrained by light when surrounded by *L. perenne* than by conspecifics under elevated CO_2. A greater increase in aboveground biomass in *L. perenne* under elevated CO_2 than in *P. lanceolata* and thus more shade may explain this result. However, the different climate scenarios did not have an effect on the aboveground biomass of *L. perenne* (data not shown). The down-regulation of plant defence against insect herbivores upon competition for light is a complex mechanisms and include more than just a resource trade-off (de Vries *et al.*, 2017).The mechanism that drives these patterns in levels of defence molecules under climate change should be

further investigated. We showed that climate change did not only have direct effects on the defence system of *P. lanceolata* (Fig. 3 pathway C) but also indirectly, through its impact on plant competition (Fig. 3 pathway A).

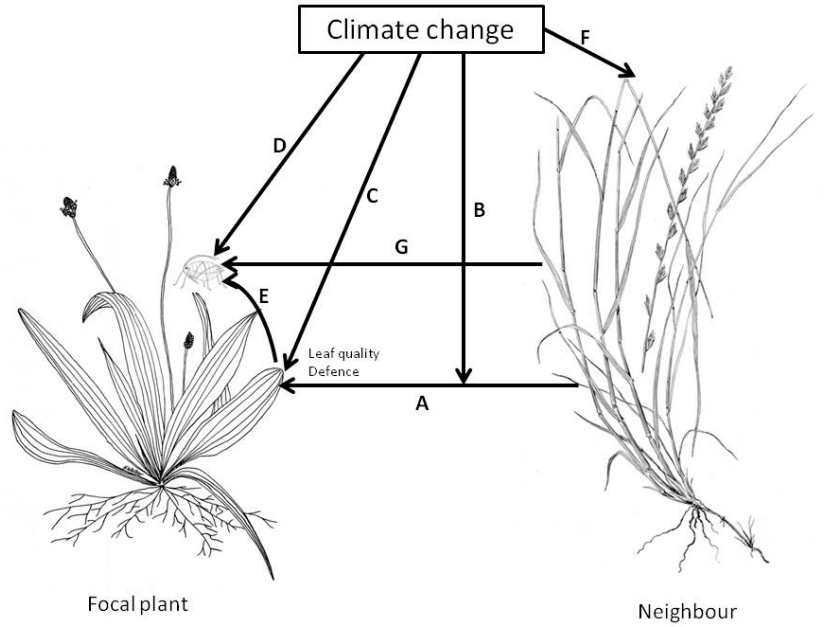

Fig. 3 Conceptual scheme illustrating the effect of a neighbour on aphid performance associated to a focal plant and the impact of climate change on these species interactions.

Plant-plant interactions did not only modify the impact of climate change on *P. lanceolata* but also the impact of climate change on aphid performance. In **chapter IV**, we found that temperature interacted with plant competition to affect aphid performance. We hypothesised that this may ascribed to an altered plant defence system under warming and interspecific competition (Fig. 3 pathway E). Indeed, we found in **chapter V** that climate change interacted with plant competition to affect the defence system (Fig. 3 pathway A). However, plant composition did not affect aphid performance. Moreover, we did not find indirect effects of climate change on aphid performance via an altered plant defence system. Although we demonstrated that climate change interacts with plant competition to affect plant defence system and aphid performance, we did not find an association between both

(Fig. 3 pathway E). However, in **chapter V**, elevated CO_2 was the most important driver of an altered plant defence system in a future climate and warming did not change the effect of elevated CO_2. In **chapter IV**, we found an interactive effect of temperature and plant competition on aphid performance. This means that this interactive effect of plant composition and temperature on aphid performance cannot be explained by an altered plant defence system. An altered plant community composition under warming might have had direct effects on aphids (Fig. 3, pathway G) (Agrawal *et al.*, 2006). Warming increased the biomass of *P. lanceolata* relative to the biomass of *L. perenne*.

The importance of plant-plant interactions in climate change experiments can also be illustrated when considering plant-herbivore interactions. Theory predicts that herbivory rates should increase exponentially with higher temperature more than primary production, reducing plant biomass at higher temperatures (Gillooly *et al.*, 2001; O'Connor, 2009; O'Connor *et al.*, 2011). In **chapter IV and V**, we showed that when taking plant-plant interactions into account, warming did not alter herbivory rates despite direct effects of warming on herbivores. This contradictory result may be due to the fact that theoretical predictions consider consumer-prey models and not plant-plant interactions (Gilbert *et al.*, 2014). In **chapter IV**, we hypothesized that reduced consumption rates at higher temperatures may be responsible for unaltered herbivory rates despite a positive effect of warming on aphid performance. This hypothesis was based on the study of Lemoine *et al*. (2014). However, declining consumption rates at high temperatures may only be relevant for leaf-chewing herbivores and not for aphids. Aphids ingest phloem sap from their hosts through stylets (narrow piercing-sucking mouthparts) and do not consume biomass (Dixon, 1998). The negative impact of aphids on their host plant is thought to be largely due to assimilate withdrawal and injection of saliva (Miles, 1999). These aphid secretions can in some cases have toxic effects and cause plant damage (Miles, 1989; Miles, 1999) but also signals associated with aphid feeding can induce a reprogramming of plant growth to the aphid's advantage (Giordanengo *et al.*, 2010). Hence, we found in chapter IV that aphid infestation reduced the biomass of *P. lanceolata*. Why did we not find increased herbivory rates at 20 °C as the aphids performed better at 20 °C compared to 17 °C? This may result from intraspecific competition for space at high density at 20 °C rather than reduced consumption rates. Are species interactions important drivers

of single species responses to climate change? Our results with a model community consisting of *L. perenne*, *P. lanceolata* and *D. plantaginea*, indicate that multispecies interactions can mediate single species responses to climate change. Therefore, failure to incorporate these interactions in experiments and models limits the ability to predict grassland vegetation responses to climate change (Gilman *et al.*, 2010; Urban *et al.*, 2016). In accordance with other authors, we highlight the need to focus more on effects of climate change on the direction and strengths of species interactions (Walther, 2010; Urban *et al.*, 2016).

Altered species interactions, together with perturbations in the abiotic environment have the capacity to change the structure of communities, because not all species in a system will respond in the same way (Sanders *et al.*, 2004; Pocock *et al.*, 2012). So far, community-level studies have shown that responses of invertebrates of different taxa to climatic and atmospheric change can be highly taxon-specific and idiosyncratic (Hamilton *et al.*, 2012; Hillstrom *et al.*, 2014; Nooten *et al.*, 2014). For instance, Hillstrom *et al.* (2014) reported that elevated CO_2 reduced the number of phloem-feeding arthropods and tended to increase numbers of chewing herbivores whereas Hamilton *et al.* (2012) found a decrease in numbers of chewing herbivores under elevated CO_2, thereby altering community composition. These and other woodland Free Air CO_2 (FACE) enrichment studies have focused entirely on relative young northern hemisphere managed Aspen plantations (Hillstrom & Lindroth, 2008; Lindroth, 2010). Experiments conducted in other habitats are necessary to gain an adequate understanding of how the invertebrate community as a whole will respond to atmospheric changes (Jamieson *et al.*, 2012; Facey *et al.*, 2014). Facey *et al.* (2017) manipulated atmospheric CO_2 in mature, native Eucalyptus woodland in Australia, in a first attempt to make more general predictions about the effect of elevated CO_2 on invertebrate communities. Elevated CO_2 decreased the overall abundance of ground-dwelling and aerial arthropods, with significant decreases in herbivore, omnivore, scavenger and parasitoid functional groups. Elevated CO_2 did not measurably affect community composition although several groups showed varying declines in abundance. Declines found in several functional groups suggest that elevated atmospheric CO_2 could potentially affect ecosystem processes such as nutrient cycling by herbivores and omnivores, as well as biocontrol by parasitoids.

6.3.4 Limitations

6.3.4.1 Design for studying effect of climate scenarios

We used an additive design in **chapter III and V** to investigate the effect of different climate scenarios on plant's intrinsic stress response. These experiments were not designed to study the effect of a future climate on grassland species apart from the stress response. The choice for this design was also the consequence of the limited number of climate chambers that was available for these experiments. Comparing the individual effect of elevated CO_2 (**chapter V**) and warming (**chapter III**) with the combined effect of warming and elevated CO_2 allowed us to see the additive effect of warming and elevated CO_2 on the stress response, respectively. However, not all responses are additive, they can be interactive or synergistic. Simply put, this means that the whole is more (or less) than the sum of the parts. A limitation of an additive design is that we cannot investigate interactive effects of warming and elevated CO_2 because we do not have both individual effects to compare with. A fully factorial design is necessary to see interactive effects of elevated CO_2 and warming on the stress response. Moreover, in **chapter III**, an effect of warming or elevated CO_2 on the drought response could also be a pure warming or elevated CO_2 effect independent of drought. We cannot exclude this because we do not have a treatment warming or elevated CO_2 without drought. The absence of an effect of temperature and elevated CO_2 before the drought does also not exclude this, because it may be that the warming or elevated CO_2 treatment needed time to induce an effect. Only a control treatment warming without drought could have precluded this.

6.3.4.2 Design for studying effect of plant-plant interactions

A substitutive design (or replacement design) was used to investigate whether plant-plant interactions modified the intrinsic stress response of grassland species to climate change. The planting density of *P. lanceolata* and *L. perenne* in monocultures versus mixtures was different but the total density was held constant. A substitutive design measure the reduction in performance caused by interspecific interactions relative to those caused by intraspecific interactions (Keddy, 1989). In this thesis, the performance of *P.*

lanceolata (and occasionally *L. perenne*) was compared in monocultures and in mixtures.

There are a few limitations with respect to the use of a substitutive design. A substitutive design relies on the assumption that individuals of interaction species are exactly equivalent at the start of the experiment which means that species must not differ in initial size to eliminate size bias (Gibson *et al.*, 1999; Jolliffe, 2000). In this thesis, we controlled for initial difference in size of *P. lanceolata* and *L. perenne* at the start of the experiment by sowing both species with a time lag of one week. Furthermore, substitutive design contains confounded species density treatments (Jolliffe, 2000). This is an important limitation of the design since the density of conspecific neighbours can increase or decrease the likelihood of damage through changes in herbivore load and feeding behaviour; these are referred to as resource concentration effects (Root, 1973) or dilution effects (Otway *et al.*, 2005), respectively. We have overcome this confounding issue by measuring aphid population characteristics per plant species.

An alternative approach is using an additive design. An additive design holds the density of one species constant across monoculture and mixtures (target species) while the density of the other species (neighbour) is varied (Gibson, 2014). It measures the reduction in performance caused by the presence of neighbours. An additive design results in a different total density between monocultures and mixtures which also can confound the effect of neighbourhood on herbivore damage.

There are many forms of interactions (i.e. any effect one plant has on another) between plants such as competition, facilitation or mutualism. In this thesis we may have labelled the interaction between *P. lanceolata* and *L. perenne* undeserved as interspecific competition while we are not sure that both species compete. Therefore, interspecific interactions is a more accurate term.

6.3.4.3 Structural equation models

We used structural equation models to disentangle direct and plant-mediated effects of warming and elevated CO_2 on aphid performance in **chapter IV and V**. However, because of the experimental set-up it was difficult to disentangle both effects of climate change and possibly a structural equation

model was not the optimal way to do it. In both SEMS in **chapter IV and V**, we used measurements on control plants (nitrogen, concentrations of primary and secondary metabolites) to predict aphid performance on different plants. Hereby we assumed that the random noise within the climate treatments is lower than between those treatments. Therefore, the lack of support for indirect effects of climate change on aphid performance (**chapter IV and V**) could be a consequence of random variation among different climate treatments that could not be attributed to the climate treatment. A better way to investigate plant-mediated effects of climate change on aphid performance is inoculating plants that have been grown at different temperatures and CO_2 conditions with insects (adapted to ambient climate conditions) under the same climate conditions (e.g. Murray *et al.*, 2013).

6.4 GENERALITY OF THE RESULTS

Through the results of this thesis we gained insight in the impact of climate change on species and ecological interactions. A thorough understanding of how climate change will affect ecological interactions would facilitate accurate predictions about community and ecosystem responses and, more importantly, could guide the effective protection of biodiversity. However, we must acknowledge a number of limitations that prevent the extrapolation of the results from our manipulation experiments to natural environments and keep us from making solid predictions.

6.4.1 Climate scenario

The future climate scenario in chapter III and V simulated a continuous 3 °C warming compared to the current climate and had a target CO_2 concentration of 620 µmol mol^{-1}. These climate manipulations were based on the IPCC-SRES B2-scenario prediction of moderate change for the year 2100 (IPCC, 2001). Based on different socio-economic scenarios, Earth System Models predict different climate scenarios. An important question is how robust our obtained results are to different climate scenarios. For instance, the life history processes of aphids increase with rising temperature to a certain threshold (Logan *et al.*, 1976; Huey & Kingsolver, 1989). The optimal temperatures and upper limits for aphids are variable but usually in the range of 20 °C to 25 °C and 25 to 30 °C, respectively (Harrington *et al.*, 1995). In Belgium, with a mean summer temperature of 17 °C, aphids are mostly living in suboptimal conditions and therefore warming (either +1 or +3) should, in principle, favour the development of aphid populations. However, in the case of *D. plantaginea*, 20 °C may be the upper thermal threshold (**chapter III**). Therefore, the magnitude of experimental warming can alter the effect of warming on this species. In reality, it is even more complex because temperature may interact with elevated CO_2 to affect aphid performance. Murray *et al.* (2013) have shown that caterpillars developed slowly when temperatures increases were minimal, as foliar quality declines in response to rising CO_2. However, significant temperature increases in the order of 4 °C could ameliorate the effects of elevated CO_2 on foliar quality by directly influencing insect physiology. We can only achieve a deeper understanding about the impact of the chosen climate scenario when bringing together these results in syntheses.

6.4.2 Spatial scale

As in most climate change experiments, we used artificially assembled grassland communities with even-aged plants planted on homogenized soil. As a consequence, our grassland communities had limited complexity and heterogeneity. However, it has been shown that soil heterogeneity modulates plant responses to climate change (García-Palacios *et al.*, 2012). Moreover, soil nutrient heterogeneity alters competitive interactions between coexisting species (Fransen *et al.*, 2001) and may thus interact with the effect of climate change on plant-plant interactions. We can overcome this problem of limited heterogeneity by bringing intact samples of established ecosystems, for instance monoliths of grassland, into climate controlled Ecotrons. That way, experiments can incorporate a natural level of heterogeneity (De Boeck *et al.*, 2015).

6.4.3 Temporal scale

In this PhD thesis, we performed short term climate change experiments. In **chapter III**, we demonstrated that a future climate induced lagged effects of drought. Such lagged effects induced by the future climate may reduce resilience of communities over time. This finding highlights that one must proceed carefully when interpreting results from short-term experiments. Indeed, contrasting effects of the climate treatment over the duration (i.e. early vs late in the experiment) has been reported (Smith *et al.*, 2015; Andresen *et al.*, 2016; Mueller *et al.*, 2016).

The duration of the experiment may also influence the results reported in **chapter V**. Induced defence in *P. lanceolata* depends on the ontogenetic stages (Bowers & Stamp, 1993). Iridoid glycosides increase as plants grow, which means that older plants are likely to be better defended against generalist herbivores than younger plants. On the one hand, low concentrations of iridoid glycosides in immature plants may be the reason why we did not find effects of altered plant defence on aphid performance. On the other hand, it is possible that changes in host-plant compounds take a number of aphid generations before changes in performance are obvious (Bezemer & Jones, 1998).

6.4.4 Community complexity

In this thesis, we used a simple model community consisting of three species: *D. plantaginea*, *L. perenne* and *P. lanceolata*. In nature, these species are part of a multitrophic community containing other plant species, herbivores and predators that cannot be ignored. Therefore, our results with two trophic levels only 'paint part of the picture'. For instance, we investigated the indirect effects of climate change on a plant's defence system only via effects on neighbouring plants. It has been shown that plant species richness and composition of the surrounding community influence the chemical defence in *Plantago lanceolata* (Mraja *et al.*, 2011). This strengthens the importance of broader community-scale experiments for a thorough understanding of the effects of a future climate on grassland vegetation. In addition, Hentley *et al*. (2014) demonstrated that the presence of a ladybird predator negated the positive effects of elevated CO_2 on aphid colonisation, and maintained populations at levels similar to those seen at ambient CO_2 concentrations. These studies, together with our results, demonstrated the importance of including multiple trophic levels and non-trophic interactions to better understand the species responses to climate change. As biological complexity of experiments is limited, enhancing the predictions requires more intensive interactions between modelling and empirical studies.

6.5 PERSPECTIVES FOR FUTURE RESEARCH

The results of this PhD thesis contribute to the knowledge on effects of climate change on grassland species and species interactions. This knowledge is a good foundation to continue further research. Below, I propose some general and specific suggestions for further research that are of particular interest in the light of the result of this PhD thesis.

While there is a wealth of information on plant responses and insect herbivore responses to warming and elevated CO_2, experimental research on the combined effect of warming and elevated CO_2 is still rare. The results of this PhD and the few other studies show that responses to simultaneously warming and elevated CO_2 often cannot be interpreted from single factor responses (Zvereva & Kozlov, 2006; Xu *et al.*, 2013). Thus, I consider, as do others (Tylianakis *et al.*, 2008; Wu *et al.*, 2011; Robinson *et al.*, 2012; e.g. Dyer *et al.*, 2013), that future research should focus on the combined effect of elevated CO_2 and warming and the interaction among them.

There are several reasons why we should focus on long-term climate change experiments. Firstly, in **chapter II** we showed that combined warming and elevated CO_2 can induce lagged effects of drought. This emphasizes the need for long term climate change experiments to see whether the resilience of grassland communities eventually reduces after a drought event. Secondly, long-term experiments are also more likely to reveal how a new community may develop as we (**chapter IV**) and other studies have shown that climate change alters species compositions (Harte & Shaw, 1995; Kardol *et al.*, 2010). Thirdly, we did not find indications of direct and indirect effects of simultaneously warming and elevated CO_2 on aphid performance (**chapter V**). As mentioned earlier, it is possible that several generations are necessary to see effects of a future climate on aphid performance. Finally, long-term experiments are important to provide input parameters for ecosystem models (Andresen *et al.*, 2016).

In **chapter V**, we demonstrated that climate change enhanced the induced defence system of *D. plantaginea*. This may have important implications for plant-insect herbivore interaction as induced defence can decrease herbivore damage. It is clear that climate change may also have indirect effects on herbivore performance not only due to altered tissue quality but also due to altered plant defence system. Despite this, the impact of climate change on

plant defence system and herbivore performance is often investigated separately. More attention must be paid to integrate both aspects together into climate change experiments. Furthermore, if we want to make accurate predictions of aphid population responses to climate change we should quantify different life history traits and not only aphid abundance.

In general, in this PhD thesis we found little evidence for indirect effects of climate change on aphid performance via altered tissue quality. However, it has been shown that altered tissue quality under climate change may affect other insect herbivores such as caterpillars (Murray *et al.*, 2013; Jamieson *et al.*, 2015). Whole-tissue chemistry could be a poor index of nutritional value for aphids since aphids depend more on the soluble amino acids in the phloem (Schoonhoven *et al.*, 2005). Despite the difficulty in sampling, it is advised to investigate pure phloem sap to explain effects of climate change on aphid performance.

In **chapter IV and V**, we demonstrated that species interactions can mediate single species responses to climate change. Therefore, as mentioned earlier, future work should focus on community-scale experiments for a thorough understanding of the effects of climate change. An important next step is introducing natural enemies in the system of plant-herbivore interactions. For instance, introducing ladybirds in our three species model community will be important for making more realistic predictions about community responses to climate change. Nevertheless, models will be necessary to predict the responses of communities and ecosystems to climate change because they work at a greater scale than can be achieved in field or laboratory studies. However, data from experimental studies are still necessary to inform them. Therefore, more attention should be paid in combining empirical and theoretical research.

6.6 GENERAL CONCLUSION

From this PhD thesis, we conclude that a future climate with combined warming and elevated CO_2 partly alleviated the biotic stress impact on grassland species. We found that some changes in plant metabolism that occur in a future climate may improve plants' protection against biotic stress. Herbivory increases both the constitutive and induced defensive (secondary) metabolites, which reduced the palatability of the plant tissue for insect herbivores. However, the altered plant metabolism under stress in a future climate did not translate into altered plant biomass production immediately after the stress event, though. A future climate did also not alter the impact of drought at the level of biomass production. Nevertheless, combined warming and drought, with or without elevated CO_2 induced higher senescence and mortality of *L. perenne* long after drought ended, while no such lagged effects were apparent in the current climate. *P. lanceolata* also exhibited post-drought lagged effects on senescence and mortality, but only under combined warming and elevated CO_2.

In general, this PhD provides strong evidence that interspecific plant interactions can mediate species responses to climate change. Therefore, we should take species interactions into account if we want to understand the effect of a future climate on vegetation dynamics. Future experimental research and models should focus more on communities instead of single species responses to climate change.

7 SUMMARY

Wherever they grow, plants are continuously exposed to stress factors of varying nature. Due to the current climate change, these stress factors will in the near future occur under greatly modified atmospheric conditions, with higher CO_2 concentrations and higher average air temperatures. This raises the question whether plants will exhibit the same stress response under these conditions. The uncertainty regarding stress responses is even greater in natural, multi-species communities than in single species. In multi-species communities, a future climate can modify the direction and the intensity of species interactions (such as plant-plant interactions). As a result, the direct effects of a future climate on the species-specific stress response might be strengthened or (partly) cancelled out. The purpose of this thesis was to investigate the direct and indirect (via altered plant-plant interactions) effects of a future climate on the stress responses of grassland species. We focused on the abiotic stressor drought and the biotic stressor herbivory.

Prior to experimental studies on the topics presented above, we examined how specific environmental conditions and the plants' stomatal response affect leaf temperatures and the potential for heat stress by using both an energy balance model and field data (**chapter II**). We found that at the same air temperature, specific atmospheric conditions can cause leaf temperatures fluctuations of 10 °C (for narrow leaves) to even 20 °C (for big broad leaves), depending on plant water status. Therefore, heat waves characterized by extreme air temperatures may pose little plant danger under some atmospheric conditions, while less high air temperatures may be lethal to plants in other cases. Our results can help ecologist and agronomists to predict when the probability of heat stress is most likely.

In a first part, by making use of a semi-field experiment we investigated whether elevated CO_2 and warming could alter the drought response of grassland monocultures and mixtures composing of *Lolium perenne* and *Plantago lanceolata* (**chapter III**). We also examined post-drought lagged effects and whether elevated CO_2 and warming altered these. We demonstrated that warming aggravated the drought impact of *L. perenne* and elevated CO_2 only partly alleviated the stress impact caused by warming and

drought. Contrary to what was found with *L. perenne*, a future climate did not alter the drought response of *P. lanceolata*. Furthermore, we showed that plant-plant interactions did not have an impact on the drought response of *L. perenne* and *P. lanceolata* in a future climate. Remarkably, combined warming and drought, with or without elevated CO_2 induced higher senescence and mortality of *L. perenne* long after the drought ended, while no such lagged effects were apparent in the current climate. *P. lanceolata* also exhibited post-drought lagged effects on senescence and mortality, but only under combined warming and elevated CO_2.

In a second part (**chapter IV and V**), by making use of a laboratory and semi-field experiment, we investigated the impact of warming, elevated CO_2 and combined warming and elevated CO_2 on a model community consisting of three species: *Dysaphis plantaginea* feeding on *P. lanceolata*, and a heterospecific neighbouring plant species, *L. perenne*. The aphid does not feed on *L. perenne*. We showed that a future climate altered the leaf quality and enhanced both the systemic and induced defence system of *P. lanceolata* against aphids. Notwithstanding these effects of a future climate on the host plant, the host plant did not have an impact on aphid performance. However, under laboratory conditions experimental warming affected aphid performance directly in non-linear manner. Aphids performed best at moderate warming, where they grew faster, had a shorter generation time and grew larger. Nevertheless, a future climate with warming and elevated CO_2 did not have direct effects on aphid performance under semi-natural conditions.

Next to these responses of *P. lanceolata* and *D. plantaginea* to a future climate, we demonstrated that plant-plant interaction can mediate these species responses. Plant-plant interactions affected aphid performance through an interaction with temperature and influenced the effect of combined warming and elevated CO_2 on leaf quality and the defence system of *P. lanceolata*. We found that interspecific plant competition neutralized the effect of elevated CO_2 on the defence molecules of *P. lanceolata*. In addition, taking plant-plant interaction into account, we showed that the interaction strength between plants and herbivores did not alter in a future climate despite effects on different components of plant-herbivore interaction.

From the result of this PhD thesis, we conclude that a future climate with combined warming and elevated CO_2 partly the alleviated biotic stress impact on grassland species. We found that some changes in plant metabolism that occur in a future climate may improve plants' protection against biotic stress. However, the altered plant metabolism under biotic stress in a future climate did not translate into altered plant biomass production immediately after the stress event, though. A future climate did also not alter the impact of drought at the level of biomass production. Nevertheless, a future climate with drought induced higher senescence and mortality long after drought ended, while no such lagged effects were apparent in the current climate. Plant-plant interactions play an important role in determining the stress response of species in a future climate and cannot be ignored. Therefore, future experimental research and models should focus more on communities instead of single species responses.

8 SAMENVATTING

In de natuur worden planten regelmatig blootgesteld aan stressfactoren van uiteenlopende aard. Door de huidige klimaatverandering zullen in de nabije toekomst deze stressomstandigheden voorkomen in een gewijzigde atmosferische omgeving met hogere CO_2 concentraties en hogere gemiddelde luchttemperaturen. De vraag dringt zich op of planten die groeien in deze veranderde omgeving nog dezelfde stressrespons zullen vertonen. De onzekerheid met betrekking tot de stressrespons in realistische gemeenschappen die meerdere soorten omvatten is nog groter, in vergelijking met de individuele stressrespons. Een toekomstig klimaat kan namelijk in gemeenschappen die meerdere soorten omvatten, de richting en de sterkte van de interacties tussen soorten (bv. plant-plant interacties) veranderen. Deze indirecte effecten van toekomstig klimaat kunnen als gevolg de directe effecten op de individuele stressrespons versterken of belemmeren. In deze thesis onderzochten we de directe en indirecte effecten (via gewijzigde plant-plant interacties) van een toekomstig klimaat op de stressrespons van graslandsoorten. We focusten op de abiotische stressor droogte en de biotische stressor herbivorie.

Voorafgaand aan de experimentele studies om bovenstaande onderzoeksvragen te beantwoorden, onderzochten we hoe specifieke omgevingscondities en de stomatale respons van planten de bladtemperatuur kunnen beïnvloeden tijdens een hittegolf met behulp van een energiebalans model en veldonderzoek (**hoofdstuk II**). We toonden aan dat bij dezelfde luchttemperatuur specifieke atmosferische condities ervoor kunnen zorgen dat de bladtemperatuur fluctueert tussen 10 °C (smalle bladeren) of zelfs 20 °C (brede bladeren) afhankelijk van de waterstatus van de plant. Hierdoor kunnen hittegolven die gekenmerkt worden door extreme luchttemperaturen weinig schade veroorzaken onder bepaalde atmosferische condities terwijl minder hoge luchttemperaturen al lethaal kunnen zijn onder andere atmosferische condities. Onze resultaten kunnen helpen te voorspellen wanneer hittestress zal optreden.

In een eerste deel werd door middel van een semi-veldexperiment de impact van verhoogde CO_2 en verhoogde temperaturen op de droogterespons van

grasland monoculturen en mengelingen van *Lolium perenne* en *Plantago lanceolata* bestudeerd (**hoofdstuk III**). We focusten ook op eventuele na-effecten van de droogteperiode en of een toekomstig klimaat deze na-effecten kan beïnvloeden. De resultaten toonden aan dat verhoogde temperatuur de droogterespons van *L. perenne* verergerde en dat verhoogde CO_2 slechts gedeeltelijk deze negatieve effecten kon compenseren. Een toekomstig klimaat had echter geen impact op de droogterespons van *P. lanceolata*. Verder hadden de plant-plant interacties geen impact op de droogterespons van *L. perenne* en *P. lanceolata* in een toekomstig klimaat. Daarnaast vonden we dat lang na de droogteperiode, een verhoogde temperatuur en droogte, in een klimaat met of zonder verhoogde CO_2, verhoogde senescentie en mortaliteit van *L. perenne* veroorzaakte. Ook *P. lanceolata* vertoonde na-effecten van de droogteperiode maar alleen wanneer verhoogde temperatuur en verhoogde CO_2 gecombineerd werden. Een huidig klimaat veroorzaakte geen dergelijke na-effecten van de droogteperiode.

In een tweede deel van deze thesis onderzochten we, aan de hand van een laboratorium en een semi-veldexperiment, de impact van verhoogde temperatuur, verhoogde CO_2 en het gecombineerde effect van beiden op een modelgemeenschap bestaande uit drie soorten: *Dysaphis plantaginea* die zich voedt met *P. lanceolata*, en een heterospecifieke buur, *L. perenne*. Deze laatste plant is geen gastheerplant voor de bladluis. We toonden aan dat een toekomstig klimaat de bladkwaliteit wijzigt en de systemisch en de geïnduceerde defensie van *P. lanceolata* tegenover bladluizen versterken. Niettegenstaande een toekomstig klimaat de samenstelling van de gastheerplant veranderde, had de gastheerplant geen impact op de bladluizen. Echter, verhoogde temperaturen hadden een direct effect op de bladluis in een laboratorium experiment maar wel op een niet lineaire manier. De bladluizen presteerden het best bij een gematigde temperatuur; ze groeiden sneller, hadden een kortere generatietijd en waren ook groter. Daarentegen vonden we geen direct effect van een toekomstig klimaat met verhoogde temperatuur en verhoogde CO_2 op de bladluizen in een semi-veldexperiment.

Bovenop deze effecten van een toekomstig klimaat op *P. lanceolata* en *D. plantaginea* demonstreerden we dat plant-plant interacties de individuele respons van soorten kunnen beïnvloeden. Plant-plant interacties

interageerden met temperatuur om zo de bladluizen te beïnvloeden. Daarnaast hadden ze ook een impact op de gecombineerde effecten van verhoogde temperatuur en verhoogde CO_2 op de bladkwaliteit en het defensiesysteem van *P. lanceolata*. Bijvoorbeeld, interspecifieke plant competitie neutraliseerde het effect van verhoogde CO_2 op de defensiemoleculen van *P. lanceolata*. Verder toonden we aan dat als we rekening houden met plant-plant interacties, de sterkte van de interactie tussen planten en herbivoren niet zal wijzigen in een toekomstig klimaat, niettegenstaande dat een toekomstig klimaat verschillende componenten van de plant-herbivore interactie beïnvloedde.

Als besluit kunnen we stellen dat een toekomstig klimaat de impact van biotische stress op graslandsoorten gedeeltelijk verminderd. In een toekomstig klimaat treden er veranderingen op in het metabolisme van planten die kunnen zorgen voor een betere bescherming tegen biotische stress. Echter, deze veranderingen in het metabolisme van planten vertaalden zich niet in een hogere biomassaproductie net na de stress. Een toekomstig klimaat had ook geen impact op het effect van droogte op de biomassaproductie. Daarentegen, droogte in een toekomstig klimaat induceerde verhoogde senescentie en mortaliteit lang na de droogteperiode. Plant-plant interacties spelen een belangrijke rol in het bepalen van de stressrespons van soorten in een toekomstig klimaat en kunnen bijgevolg niet genegeerd worden. Toekomstig experimenteel en modelmatig onderzoek zouden meer moeten focussen op gemeenschappen in plaats van op de respons van individuele soorten.

9 DANKWOORD

Het boekje is klaar. Er rest mij nog maar één ding, namelijk een aantal mensen in de bloemetjes te zetten en hen te bedanken voor hun hulp en steun die ze mij boden tijdens dit doctoraat.

Graag wou ik een eerste woord van dank richten aan mijn promotor Ivan. Je hebt mij heel veel vrijheid gegeven om zelfstandig te werken en een eigen inslag te geven aan dit doctoraat. Dankjewel voor de tijd die je nam om tijdens overlegmomenten alles zo grondig door te nemen zowel voor wat de experimenten betreft als voor de artikels. Door jouw kritische visie en interessante wetenschappelijke ideeën heb ik heel veel geleerd.

Dankjewel Dries om eveneens promotor te willen zijn! Je hebt mij de kans gegeven om het doctoraat de wending te geven die ik wou ingaan. Heel hartelijk bedankt omdat ik steeds zo snel bij jou terecht kon als ik strop zat. Tijdens de vele meetings kon jij steeds een juiste kijk op het onderzoek geven en jouw enthousiasme werkte aanstekelijk en motiverend.

Reinhart en het PLECO team wil ik bedanken voor de kans die ze mij gegeven hebben om dit doctoraat te beginnen en voor het vertrouwen dat ze hadden dat het tot een goed einde zou komen.

De jury (Martijn Bezemer, Martijn Vandegehuchte, Han Asard en Ivan Janssens) wil ik bedanken voor de tijd die ze vrijgemaakt hebben om dit werk grondig na te lezen en voor hun constructieve feedback. Ze hebben mijn doctoraat allicht opgetild tot een hoger niveau.

Een speciaal woord van dank gaat naar Joke en Kim van PLECO. Dankjewel om mij wegwijs te maken in het reilen en zeilen van de N-site, in de opstart van experimenten en in het onderwerp van dit doctoraat. Ook de andere collega's van PLECO wil ik bedanken voor de hulp bij het veldwerk (zoeken naar bladluizen als een speld in een hooiberg is niet gemakkelijk), de fijne babbels, de leuke weekendjes, cookie-time,… Het was een zeer aangename werkplek. Nadine, Nele, Marc, Fred en Eddy wil ik bedanken voor hun technische ondersteuning.

De collega's van de voorbije jaren op TEREC wil ik bedanken voor de fijne momenten op het werk, maar ook daarbuiten op congres of op de jaarlijkse veldstage aan de kust in juni. Hans wil ik bedanken voor de hulp tijdens het veldwerk. Een speciaal woordje van dank gaat naar mijn bureaugenoten Katrien, Annelies en Jasmijn. Dankjewel voor al jullie advies en de leuke babbels over koetjes en kalfjes.

Dikke merci aan alle vriendinnen en vrienden voor de steun, de interesse in mijn werk en het begrip tijdens de drukkere periodes. Dankjewel voor de momenten van plezier en ontspanning, ze vormden een welgekomen afleiding.

Heel graag wil ik mijn ouders, mijn zussen en schoonbroers bedanken voor de enorme steun die ik mocht ervaren, de stimulans om steeds verder te doen, de overlegmomenten en de hulp tijdens het veldwerk. Zeker tijdens het afwerken van dit doctoraat waren jullie van onschatbare waarde. Bram, dankjewel voor het nalezen van deze thesis. De oma's wil ik enorm bedanken voor de talloze babysits en de hulp in het huishouden, zodanig dat ik dit boekje kon afwerken. De laatste loodjes waren zeer zwaar, ook voor jullie. Dankjewel dat ik altijd op jullie kon rekenen.

Lieve Philip, dankjewel om mij steeds te blijven steunen, het geduld, het luisterend oor en de vele hulp die ik van jou gekregen heb. Er zijn wel leukere dingen dan op een zaterdag bladluizen te verwijderen. Dankjewel voor het begrip dat tijdelijk alles in het teken stond van het afwerken van mijn doctoraat. Dankjewel om met de kindjes op uitstap te gaan zodanig dat ik rustig kon werken. Dankjewel om eten te maken voor ons allen zodanig dat ik maar mijn voeten onder tafel moest steken. Dankjewel om er steeds voor mij te zijn!

Lieve kleine Lasse en Wannes, jullie mogen zeker niet vergeten worden. Jullie hielpen mij het meest door de zware laatste loodjes. Een glimlach, een dikke knuffel en kus en ik was plots al mijn zorgen vergeten. De zorg voor jullie plaatste alles in een goed perspectief. Wat zullen jullie blij zijn dat mama weer tijd heeft om te spelen.

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